

THE HUMAN ANTIBODY RESPONSE TO DENV2  
INFECTION AND VACCINATION

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## **ABSTRACT**

Emily Newman Gallichotte: The Human Antibody Response to DENV2 Infection and  
Vaccination  
(Under the direction of Aravinda de Silva and Ralph Baric)

Dengue viruses (DENVs) are mosquito-borne flaviviruses that are estimated to infect 390 million people each year. Dengue is a major global public health concern because people infected with the virus can develop dengue fever or severe dengue hemorrhagic fever and shock syndrome. Vaccines offer the best hope for controlling the current global DENV pandemic. The major goal of my thesis project was to define the properties of neutralizing and protective human antibodies stimulated by natural DENV infections and the leading live attenuated DENV vaccines.

There are four antigenically distinct DENV serotypes, named DENV1 through DENV4. For my studies, I focused on DENV2 as a model to understand human protective immunity following infection or vaccination. Following natural DENV2 infections, individuals generate strongly neutralizing DENV2 serotype-specific antibodies, which provide protection against subsequent DENV2 infections. I characterized the properties and specific epitopes of multiple human DENV2 serotype-specific strongly neutralizing monoclonal antibodies, and discovered two major antigenic sites on domain III and domain I of the DENV2 envelope protein. Additionally, I found that the majority of DENV2 serotype-specific polyclonal antibodies present in immune sera also target quaternary epitopes as defined by these DENV2 monoclonal antibodies.

I sought to determine if DENV vaccination is able to elicit the same types of DENV2 antibodies implicated in protective immunity following natural infections. I observed that two different live DENV vaccines were able to elicit antibodies targeting epitopes similar to those targeted by antibodies following natural DENV2 infections, suggesting that these vaccines might be protective against DENV2 challenge.

Investigators studying DENV pathogenesis and vaccines had previously focused on the functional properties (neutralization) of human antibodies, with little consideration of the actual epitopes and mechanisms of protective immunity. Recent results from vaccine trials indicate that the mere presence of antibodies capable of neutralizing DENV in cell culture assays was not sufficient for protection from WT DENVs. My studies provide an in-depth view of the molecular specificity of serotype-specific human antibodies that neutralize DENVs. As a result of my thesis work, it is now possible to go beyond neutralizing antibodies and define the fine specificity and other properties of serotype-specific antibodies induced by infection or vaccination. My studies shed light on the protective immune response to DENV infections, which can ultimately be harnessed to evaluate current DENV vaccines, and improve the design of the next-generation of DENV vaccines.



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## LIST OF ABBREVIATIONS

AA – amino acid

Ab – antibody

ADE – antibody dependent enhancement

cDNA – complementary deoxyribonucleic acid

CR – cross-reactive

cryo-EM – cryo-electron microscopy

CYD – chimeric yellow fever dengue

CYD-TDV – chimeric yellow fever dengue-tetravalent dengue vaccine

DC-SIGN – dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DENV – dengue virus

DNA – deoxyribonucleic acid

E – envelope

*E. coli* - *Escherichia coli*

ED – envelope domain

EDE – envelope dimer epitope

EDI – envelope domain I

EDI/II – envelope domain I and II

EDII – envelope domain II

EDIII – envelope domain III

ELISA – enzyme-linked immunosorbent assay

FC – fragment crystallizable

FFU – focus forming unit

FRNT – focus reduction neutralization test

FRNT<sub>50</sub> – focus reduction 50% neutralization titer

HIV – human immunodeficiency virus

HMAb – human monoclonal antibody

IgG – immunoglobulin G

IgM – immunoglobulin M

LAV – live attenuated vaccine

LLPC – long lived plasma cell

MAb – monoclonal antibody

MBC – memory B-cell

NAb – neutralizing antibody

Neut – neutralization assay

Neut<sub>50</sub> – 50% neutralization titer

NHP – non-human primate

NHS – normal human sera

NIH – National Institutes of Health

NS – non-structural protein

OD – optical density

PAb – polyclonal antibody

PCR – polymerase chain reaction

pr – precursor protein

prM – precursor membrane protein

rDENV – recombinant dengue virus

rE – recombinant envelope protein

rEDIII – recombinant envelope domain III protein

RFLP - restriction fragment length polymorphism

RNA – ribonucleic acid

RT-PCR – reverse transcriptase polymerase chain reaction

TS – type-specific

WNV – West Nile virus

WT – wildtype

YFV – yellow fever virus

ZIKV – Zika virus

## **CHAPTER 1 – The Molecular Specificity of the Human Antibody Response to Dengue Virus Infections**

### **1.1 Summary**

Dengue viruses (DENV) are mosquito-borne positive sense RNA viruses in the family Flaviviridae. The four serotypes of DENV (DENV1, DENV2, DENV3, DENV4) are widely distributed and it is estimated over a third of the world's population is at risk of infection (1). While the majority of infections are asymptomatic, DENV infection can cause a spectrum of disease, from mild flu-like symptoms, to the more severe DENV hemorrhagic fever and shock syndrome (2). Over the past 20 years, there have been intense efforts to develop a tetravalent live-attenuated DENV vaccine (3). The process of vaccine development has been largely empirical, because effective live attenuated vaccines have been developed for other flaviviruses like yellow fever and Japanese encephalitis viruses. However, recent results from Phase III live attenuated DENV vaccine efficacy trials are mixed with evidence for efficacy in some populations but not others (4). In light of unexpected results from DENV vaccine trials, in this chapter we will review recent discoveries about the human antibody response to natural DENV infection and discuss the relevance of this work to understanding vaccine performance.

### **1.2 DENV Structure**

The DENV genome encodes a single open reading frame that is translated into a polyprotein. Viral and host proteases cleave the polyprotein into three structural and seven non-

structural viral proteins. The structural envelope protein (E) contains three domains, domain I (EDI), domain II (EDII) and domain III (EDIII) (5). Two envelope monomers come together in a head-to-tail orientation, forming the E dimer (Figure 1.1). Three E dimers form the dimer raft, and 30 dimer rafts cover the surface of the DENV virion in icosahedral orientation with both 3-fold and 5-fold axes of symmetry. Domain II contains the hydrophobic fusion peptide, which mediates fusion between the virus and host cell membrane. To prevent fusion with the host-membrane during egress from infected cells, the pre-membrane (prM) protein covers the fusion loop. As the virus moves through the endosome, pH changes triggers the host protease furin to cleave the prM protein (6). As the virus is released from cells, cleaved prM dissociates from the virion. This process is inefficient however, leaving a heterogeneous population of fully mature (no prM present), fully immature (containing prM), and partially mature virions (7). While cell culture grown virus shows a spectrum of maturation states, it is now clear that the overall maturation state of virions can vary between strains and even between different preparations of the same strain (8). As we discuss later, maturation state can influence the ability of some antibodies to bind and neutralize DENV and other flaviviruses.

### **1.3 Antibody Response to DENV Infection**

The basic kinetics of the DENV specific Ab response, the timing of IgM and IgG Ab development and the timing of neutralizing antibody (Nab) development have been well understood for many years (9-11). In brief, individuals with no prior immunity to DENVs mount a primary antibody response that includes a specific IgM response followed by a durable IgG response. The initial IgG response contains different types of antibodies, including serotype cross-reactive neutralizing antibodies, serotype cross-reactive non-neutralizing antibodies, and serotype-specific neutralizing antibodies (12). The serotype cross-reactive neutralizing antibodies may provide immediate protection to subsequent infection with any of the DENV serotypes, but these antibodies wane over the course of a year. DENV serotype-specific

neutralizing antibodies and some cross reactive poorly neutralizing antibodies are maintained for decades following infection and appear to protect against subsequent re-infection with the same serotype, but do not protect against the other serotypes (Figure 1.2). Conversely, cross-reactive antibodies not only are non-protective, but can enhance subsequent infection via a mechanism known as antibody dependent enhancement (ADE) whereby non-neutralizing antibodies bind the virus and the antibody-virus complex is taken up by cells via FC-receptor mediated endocytosis (13). Although ADE is poorly understood, the response is important in natural infection and vaccine development but will not be discussed in this review. Readers are recommended to refer to these earlier reviews for additional information on ADE and DENV (13-15).

#### **1.4 Methods to Study the Molecular Specificity of Human Antibodies to DENVs**

A variety of techniques have been used to map the viral epitopes targeted by polyclonal antibodies in human immune sera and monoclonal antibodies (MAbs) isolated from dengue patients (Figure 1.3). Traditionally, to study DENV-specific MAbs, peripheral blood B-cells from DENV immune donors are transformed and clones secreting DENV-reactive MAbs are fused with myeloma cell lines to generate human hybridomas producing the MAb of interest (16, 17). Recent advances in single-cell-sequencing has allowed individual IgG heavy and light chains from the same cell to be sequenced, cloned and recombinantly expressed (18, 19). The properties and specificity of these MAbs can then be determined using binding assays to recombinant DENV proteins (rE and rEIII) and whole virions, and neutralization assays, as well as by solving high-resolution structures of the MAb bound to viral antigen. Once a putative MAb epitope has been identified, recombinant viruses with point mutations at the region of interest can be used to confirm and further refine the epitope. Importantly, these studies have revealed that most DENV neutralizing epitopes are created by presentation of discontinuous residues that are brought together in tertiary and quaternary structures. Additionally, our group



has shown that the discontinuous residues that comprise these complex epitopes can be transplanted to a different serotype to generate chimeric DENVs that encode neutralizing epitopes from multiple DENV serotypes, and which can be used to map and confirm the binding and neutralization of individuals MABs (20, 21).

Polyclonal sera contains a complex mixture of DENV-specific IgG antibodies, those that are neutralizing or non-neutralizing, and those that are specific to a serotype or cross-reactive to multiple serotypes (Figure 1.2). Depletion assays can be used to determine the percentage of neutralizing serotype-specific antibodies to neutralizing cross-reactive antibodies (Figure 1.3A). To remove cross-reactive Abs, primary infection sera can be incubated with beads coated with a heterologous serotype (e.g. a primary DENV2 sera can be incubated with DENV1/DENV3/DENV4-coated beads). Cross-reactive Abs will bind to the virus on the beads and be pelleted out, leaving only DENV2 serotype-specific Abs. Neutralization assays using depleted sera allow one to calculate the fraction of neutralization due to serotype specific Abs, relative to the total neutralization coming from both serotype-specific and cross-reactive Abs (22-24). These depletion techniques, in addition to use of epitope transplant chimeric rDENVs described above, has allowed us to study the amount of polyclonal antibodies targeting epitopes represented by individual MABs (21, 23).

### **1.5 Molecular Specificity of Neutralizing MABs from Primary Cases**

The most striking feature of primary DENV infections is the rapid clearance of the virus and the maintenance of serotype-specific neutralizing Abs in the serum in most individuals for decades if not longer. Recent studies have only begun to define the molecular specificity of human B-cells and antibodies responsible for durable type-specific neutralization and protection. The envelope protein is the major antigenic protein; the majority of DENV-specific antibodies target E (5). Traditionally human monoclonal antibodies (MABs) have been screened based on

their ability to bind recombinant envelope monomeric protein (rE). This has biased our study of MAbs to those that recognize simple epitopes; their epitopes are contained within a single E protein. Recent work by many groups has expanded this study to screen antibodies based on their ability to bind the entire DENV virion (25, 26). This work has identified antibodies from each serotype that recognize complex, quaternary epitopes, which are assembled from higher-order structures that span across monomers, dimers, rafts, or require specific angles or presentation of E, that is only present on the intact virus (Figure 1.4). Additionally, it has been found that while antibodies using simple epitopes can be neutralizing, it is the antibodies recognizing complex epitopes that are ultimately responsible for polyclonal neutralization (21, 27, 28). Antibodies recognizing quaternary epitopes are not unique to DENV; West Nile Virus (WNV) and Zika Virus (ZIKV) have also been shown to generate human MAbs recognizing similar complex epitopes (29-32).

## **1.6 Differences in Neutralizing MAb Epitopes Across Serotypes**

While the E protein is around 80% conserved across all four DENV serotypes, it appears the different serotypes may use different immunodominant epitopes (Figure 1.4). Unlike anti-DENV mouse MAbs that predominantly target EDIII (33, 34), many human MAbs use EDI, EDII, and the EDI/II hinge region in their epitopes. For example, DENV1 MAbs 1F4 and 14C10 use a quaternary EDI epitope that spans across dimers within the raft and can reach into EDIII of the neighboring dimer (35, 36). Interestingly, this epitope is highly similar to a human WNV MAb CR4354, suggesting WNV might use a similar immunodominant epitope (32). The DENV3 MAb 5J7 targets an epitope centered around the EDI/II hinge region that is dependent on a specific hinge angle, only present on intact virion (37). Recent work has identified human DENV4 MAbs that target a similar epitope (23). Interestingly, DENV2 MAbs appear to use an epitope distinct to the EDI/EDII region, instead centering around EDIII (21, 38). Our understanding of immunodominant epitopes for each serotype is informed by only a handful of monoclonal

antibodies from a few immune individuals. To fully define the boundaries of the polyclonal neutralizing epitopes against each serotype, additional antibodies from more individuals will need to be studied.

### **1.7 Cryptic Epitopes**

The majority of human epitopes studied are present on the surface of the intact virion. Some studies have identified mouse MAbs that target cryptic epitopes not readily accessible on the surface of the virus. However, at elevated temperature these cryptic epitopes are transiently displayed, allowing antibody binding and neutralization (39). Recent studies suggest that there are antibodies present in human immune sera that also target these cryptic epitopes, potentially allowing the virus to be neutralized when it is under specific conditions exposing these epitopes (39). However, we need to identify human MAbs that target these cryptic epitopes and determine their role and importance in neutralization and protective immunity.

### **1.8 Other Flaviviruses – Zika Virus MAbs**

With the emergence of Zika virus (ZIKV), approaches developed for DENVs have been extended to isolate and map the human antibody response to ZIKV (29-31). Multiple groups have generated human ZIKV MAbs and characterized their epitopes on E. Individual MAbs have been identified with epitopes that span the whole E protein, but, similarly to DENV, the strongest neutralizing MAbs target quaternary epitopes only present on the intact ZIKV virion. These quaternary ZIKV epitopes are similar to previously identified quaternary DENV epitopes that are centered around the EDI/II hinge region, span across E monomers within the dimer, or span across dimers (29-31).

## **1.9 Mapping the Molecular Specificity of the Polyclonal Serum Neutralizing Antibody Response**

While MAbs are isolated or generated from memory B-cells, circulating polyclonal antibodies come from plasma cells (40). The memory B-cell derived human MAbs can be used as tools to interrogate the properties and specificity of the more complex polyclonal serum antibody response (Figure 1.5). Work by multiple groups have shown that individual MAbs can be representative of the anti-DENV B-cell repertoire, polyclonal Abs from the same individual, and polyclonal Abs across other naturally infected and vaccinated individuals, confirming the importance of studying individual monoclonals (21, 23, 41). Importantly, depletion assays have revealed that after primary DENV infections, the majority of polyclonal neutralization comes from serotype-specific antibodies, not cross-reactive ones (22-24). Additionally, we have found that epitopes defined by individual MAbs that are complex and quaternary, are representative of the polyclonal epitopes targeted by neutralizing serotype-specific antibodies (27, 28). With the rapid emergence of ZIKV, similar techniques as described above were applied to dissecting the antibody response to ZIKV infection. Multiple groups have found that strongly neutralizing ZIKV MAbs target complex quaternary epitopes (29-31). Additional work using depletion assays, has identified that primary ZIKV infections can result in ZIKV specific Abs, despite populations of Abs that cross-neutralize DENV (42).

## **1.10 Molecular Specificity of Neutralizing Antibodies Following Secondary DENV Infection**

Individuals experiencing secondary DENV infections with a new serotype develop a neutralizing and protective antibody response that is fundamentally different from a primary infection-induced response. People with known sequential infections with two different DENV serotypes have type-specific antibodies to serotypes of infection and a new population of durable serotype-cross neutralizing antibodies that are also effective against serotypes not

encountered by the person (43). Human cohort studies in dengue-endemic countries have also established that tertiary infections are nearly always mild or inapparent, implicating a protective role for these broadly cross-neutralizing antibodies that develop after a second DENV infection (44). Figure 1.6 presents a model to explain the evolving antibody response following sequential DENV infections with different serotypes. The model is based on the premise that low affinity DENV cross reactive memory B-cells derived from primary infections undergo antibody somatic hyper mutation and each subsequent DENV exposure selects and expands rare affinity matured clones with greater neutralization breadth and potency (24). The model is supported by recent studies demonstrating that serotype cross-reactive antibodies derived from secondary infections had stronger neutralization potencies and higher binding avidities than those derived from patients with primary infections (45-49).

While we know a lot about epitopes targeted by DENV serotype-specific neutralizing and protective antibodies, less is known about the targets of durable serotype-cross neutralizing antibodies. Several cross-neutralizing human MAbs that bind to an epitope near the bc-loop on domain II the E protein monomer have been recently described (Figure 1.7) (45, 50). Another class of serotype cross-reactive and strongly cross-neutralizing MAbs, which bind to quaternary epitopes on the E homodimer, was recently isolated from acute-phase plasmablasts in the peripheral blood of secondary DENV cases (25, 51). These MAbs, which have been designated E dimer epitope (EDE) antibodies, bind to epitopes that span domains I or III of one monomer and domain II of the adjacent monomer (Figure 1.7). It is unclear if the strongly cross-neutralizing MAbs isolated from acute-phase plasmablasts are maintained as MBCs and LLPCs and responsible for the durable cross-neutralizing antibodies observed in people after recovery from secondary infections. Additionally, there are still unknowns regarding if the order of infecting serotypes is important for the epitopes of strongly cross-neutralizing MAbs. The

molecular mechanisms leading to the evolution of cross-neutralizing antibodies from the memory B-cell pool from a primary infection are also unclear.

### **1.11 NS1 and prM MAbs**

While E is the major antigenic protein of DENV, antibodies are also generated targeting the viral proteins NS1 and prM. The host sees prM protein in several configurations. As mature viruses are released from infected cells, prM protein dissociates from the virus and is released as an antigen. Additionally, immature viruses have prM present on their surface, allowing the immune system to recognize them as part of the virus. prM antibodies are predominantly non-neutralizing enhancing antibodies; they allow non-infectious immature viruses to be taken up into cells via FC-receptor-mediated endocytosis (52).

DENV NS1 protein has many roles depending on its interactions and location (53, 54). NS1 can exist as a monomer, dimer or hexamer, and is important in viral RNA replication, viral assembly and release, and immune evasion. NS1 is secreted from infected cells primarily as a hexamer, which can bind to endothelial cells, triggering hyperpermeability, leading to vascular leakage seen in severe DENV disease (55). Clinically, levels of circulating NS1 are correlated with disease severity (56, 57). People infected with DENV make antibodies directed against NS1, but it is unclear if these are an important part of the protective immune response, or are merely a consequence of high levels of circulating viral antigen (54).

### **1.12 Mechanisms of Neutralization**

MAbs can neutralize viruses through a variety of mechanisms. MAbs have been shown to neutralize DENV by blocking attachment to host cell receptors, binding directly to the fusion-loop, binding across E proteins preventing conformational changes required for fusion, as well as via opsonization. Anti-DENV MAbs have been shown to neutralize using many of these

mechanisms (37, 38, 51, 58). DENV maturation state (amount of prM present) and virus breathing are important factors for virus neutralization. A fully immature virus (i.e. 180 copies of prM present) is non-infectious, and therefore cannot be neutralized, but a partially mature virus, can still be infectious (59). Alternatively, under certain temperature conditions, some DENV strains can undergo reversible conformational changes where the E proteins expand and contract analogous to “breathing”. These expansion and contraction changes can reveal or hide epitopes, limiting neutralization by antibodies recognizing these epitopes to specific conditions (39, 60-62). While DENV maturation and “breathing” have been studied in cell culture systems, the importance of these phenomenon in natural infection, and therefore the potential impact on antibody neutralization, is not well understood.

### **1.13 Implications for Evaluating Antibodies to DENV Live Attenuated Vaccines (LAVs)**

Recently we have learned important lessons from DENV tetravalent vaccine clinical trials. The leading tetravalent vaccine had variable efficacy depending on DENV serotype and vaccinated population (4). The vaccine had higher efficacy in DENV-primed individuals compared to DENV naïve individuals who received the vaccine, establishing the impact of immunological memory on vaccine performance (63). The population with the greatest need for a DENV vaccine is young children, the majority of whom will be DENV-naïve at vaccination. As discussed above, in people exposed to primary natural DENV infections, the neutralizing and protective antibody response is dominated by type-specific antibodies to quaternary epitopes. Therefore, in this population the success of tetravalent vaccination is likely to require balanced replication of the four vaccine viruses leading to type-specific antibodies that target quaternary epitopes in each serotype.

As discussed above, secondary DENV infections result in activation of memory B-cells and development and expansion of cross-reactive antibodies that broadly neutralize multiple

DENV serotypes, driven by the sequential infection and robust replication of two different serotypes of DENV (24). A similar mechanism is likely to be responsible for the superior performance of tetravalent LAVs in DENV-primed individuals. In a subject with pre-existing DENV-specific MBCs, even unbalanced replication of one or two vaccine components is likely to activate MBCs and expand somatically mutated higher-affinity cross-reactive clones with capacity to broadly neutralize multiple serotypes.

Immune correlates of protection and vaccine efficacy are urgently needed. For the leading DENV vaccine, the mere presence of in vitro neutralizing antibodies was not sufficient for protection because many individuals experienced breakthrough infections despite having neutralizing antibodies to the breakthrough serotype (63). The lessons we have learned from natural infections studies about the molecular specificity of human antibodies to DENV infection may also lead to more robust correlates of vaccine efficacy than mere levels of total neutralizing antibodies (3). Certainly, the reagents and tools are now available to interrogate vaccine responses in a manner similar to that we have described here for natural DENV infections.

#### **1.14 Objectives of this Dissertation**

Despite DENV being endemic in over 100 countries, with a third of the world's population at risk for infection, and almost 400 million infections yearly (1), there are still many aspects of the DENV infection that are poorly understood. Importantly, in most individuals, natural exposure to infection results in a robust adaptive immune response, which provides subsequent protection against reinfection with the same serotype. Within this immune response, we know that strongly neutralizing antibodies play a critical role in subsequent protection from reinfection, but we still do not have a complete understanding of the properties of protective antibodies and the mechanisms of antibody-mediated protection.



Early research on immunity to DENVs relied heavily on monoclonal antibodies (MAbs) generated in mice. Extensive studies with mouse MAbs identified a primary epitope in EDIII of the envelope glycoprotein recognized by strongly neutralizing antibodies (64). Subsequent studies revealed that, while this epitope is immuno-dominant in mice, only a small portion of the human serological response targets this EDIII epitope (28, 64, 65). This research revealed that the human antibody response to DENV infection is distinct from that in a mouse, and therefore to fully characterize it, we must isolate and characterize the activities of human DENV antibodies (64). Unlike mouse EDIII epitopes, it was found that human strongly neutralizing MAbs for DENV1, DENV3 and DENV4 target an epitope centered near the EDI/II hinge region (23, 27). Importantly, these are not simple epitopes present on recombinant proteins, but quaternary; they are only present on the fully assembled infectious virion (23, 27). Recent advances have shown that polyclonal serum neutralizing antibodies are also directed to quaternary structure epitopes defined using MAbs (23, 27).

Cell culture based assays can determine levels of neutralizing antibodies present in serum, and it was hoped that this could be developed as a correlate of protection, especially for developing and evaluating DENV vaccines (66, 67). The recent clinical trials from Dengvaxia, Sanofi Pasteur's tetravalent DENV vaccine, have revealed that some individuals with high levels of neutralizing antibodies experience DENV breakthrough infections (68, 69). Of note, vaccine efficacy was worst against DENV2, highlighting the weakness of the DENV2 component in eliciting a robust, protective antibody response (68). These results highlighted the discrepancy between the mere presence of neutralizing antibodies and protection against natural infection as individuals with neutralizing antibodies, experienced natural breakthrough infections. Additional work distinguishing between different populations of antibodies potentially revealed a mechanism to explain the large differences in vaccine efficacy across serotypes.

DENV polyclonal immune sera are comprised of multiple populations of antibodies, including type-specific antibodies specific to the infecting serotype, and weakly neutralizing cross-reactive antibodies (12). It is thought that cell-culture based assays can over-represent the neutralization potency by cross-reactive antibodies. Using depletion techniques we can study the contribution of each of these populations of antibodies on total neutralization (27, 70). Recent work identified that Dengvaxia vaccine sera contains large populations of DENV4 serotype-specific neutralizing antibodies, however DENV1-3 neutralization is driven primarily by cross-reactive antibodies (22). Analysis of vaccine sera from individuals who experienced breakthrough infections, versus those who did not, we can potentially determine an improved antibody and epitope based correlate of protection, by specifically measuring the quality of antibodies elicited by vaccination.

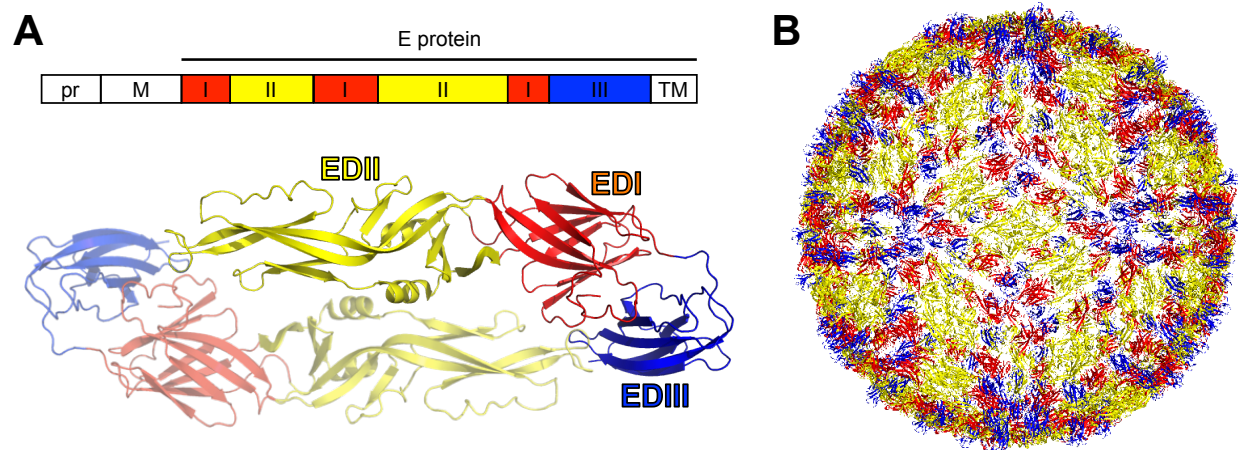
Identifying the dominant epitopes targeted by human DENV2 serotype-specific monoclonal and polyclonal antibodies is critical for refining our understanding of response to infection and vaccination. Detailed characterization of these DENV2 antibodies and the epitopes they target, will improve our understanding of the complex adaptive immune response to DENV infection, and can be harnessed to improve design and evaluation of DENV vaccines.

*We hypothesize that 1) DENV2 serotype-specific neutralizing antibodies target multiple quaternary epitopes on the envelope glycoprotein, 2) that generating a robust DENV2-specific antibody response directed to these epitopes is critical for vaccine-elicited protection, and that 3) DENV1-specific neutralizing epitopes are distinct from those of DENV2, but can be mapped and studied using similar techniques.*

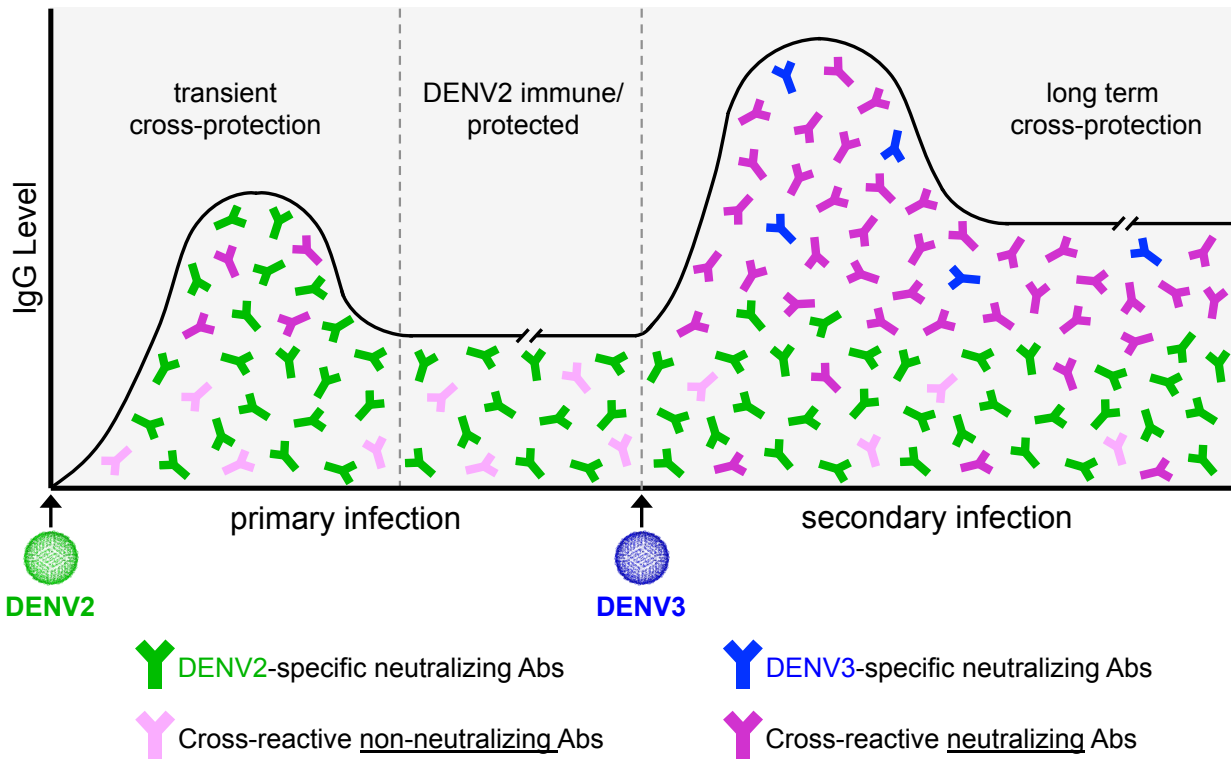
**AIM 1:** Map the epitopes of human DENV2 serotype-specific monoclonal and polyclonal antibodies using recombinant DENVs, chimeric epitope transplant viruses, monoclonal blockade/competition assays, binding and neutralization assays.

**AIM 2:** Evaluate the DENV2 serotype-specific neutralizing antibody response following tetravalent DENV vaccination, by determining the quality of DENV2 neutralizing antibodies, the epitopes they target, and whether these responses differ in individuals who experienced DENV2 breakthrough infections, versus those who did not.

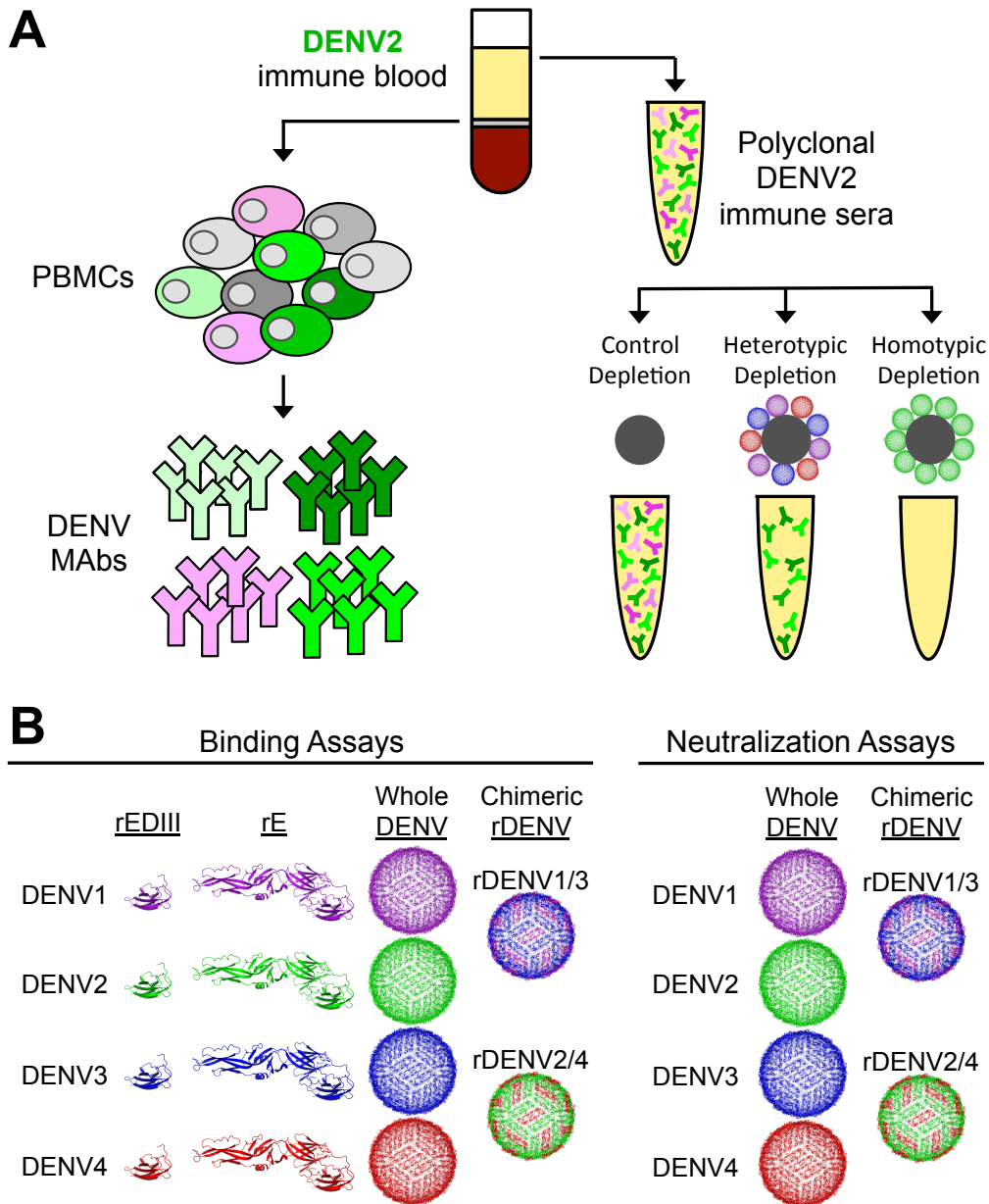
**AIM 3:** Map the epitopes of DENV1 serotype-specific monoclonal antibodies by characterizing a DENV1 infectious clone, and by using an epitope transplant viruses in binding and neutralization assays.



**Figure 1.1. Structure of DENV.** **A)** Linear schematic of DENV envelope (E) protein. DENV E protein dimer composed of two monomers with domains I, II and III colored in red, yellow and blue respectively. **B)** DENV virion structure composed of 30 rafts, each containing three E dimers.

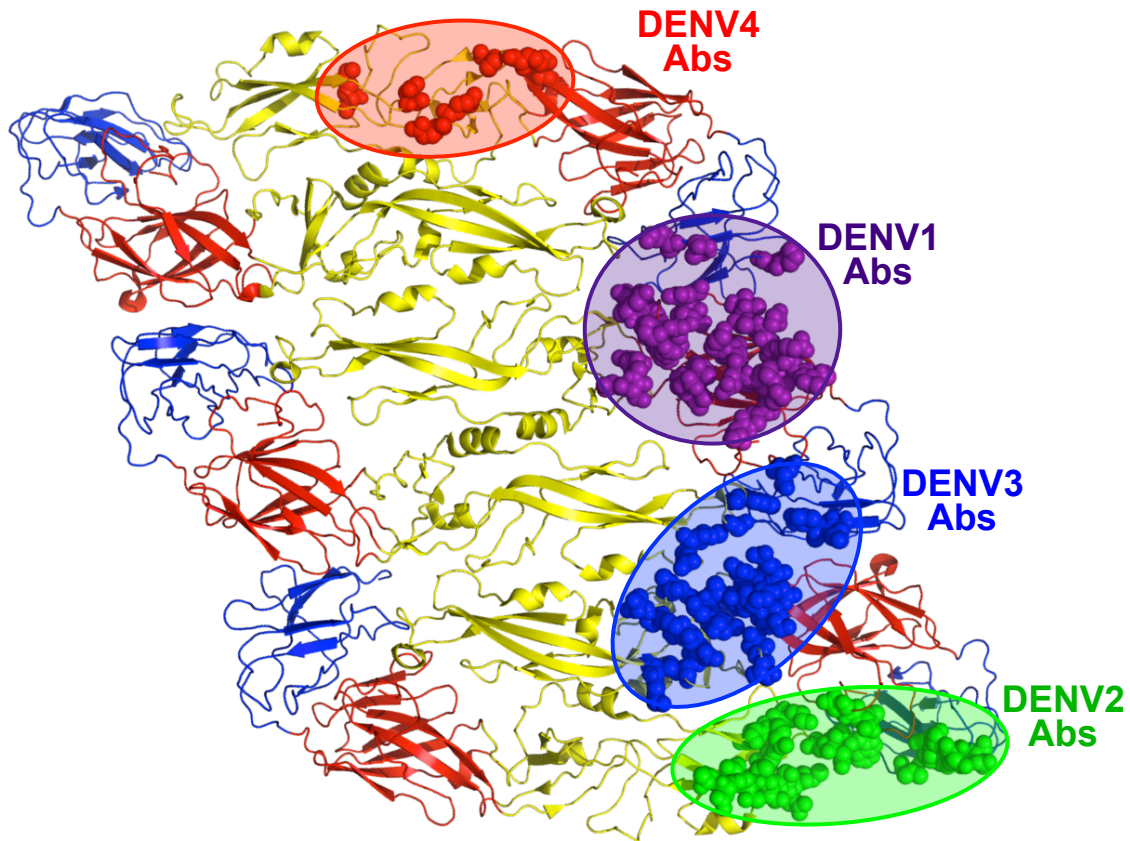


**Figure 1.2. Antibody response following DENV infection.** Following primary DENV2 infection, there is an IgG response composed of neutralizing DENV2 serotype-specific antibodies, a transient population of cross-reactive neutralizing antibodies, and long-lived cross-reactive non-neutralizing antibodies. After a secondary infection, in this case with DENV3, the cross-reactive non-neutralizing antibodies become strongly neutralizing. It is also possible to generate a new population of neutralizing serotype-specific antibodies to the second infecting serotype.



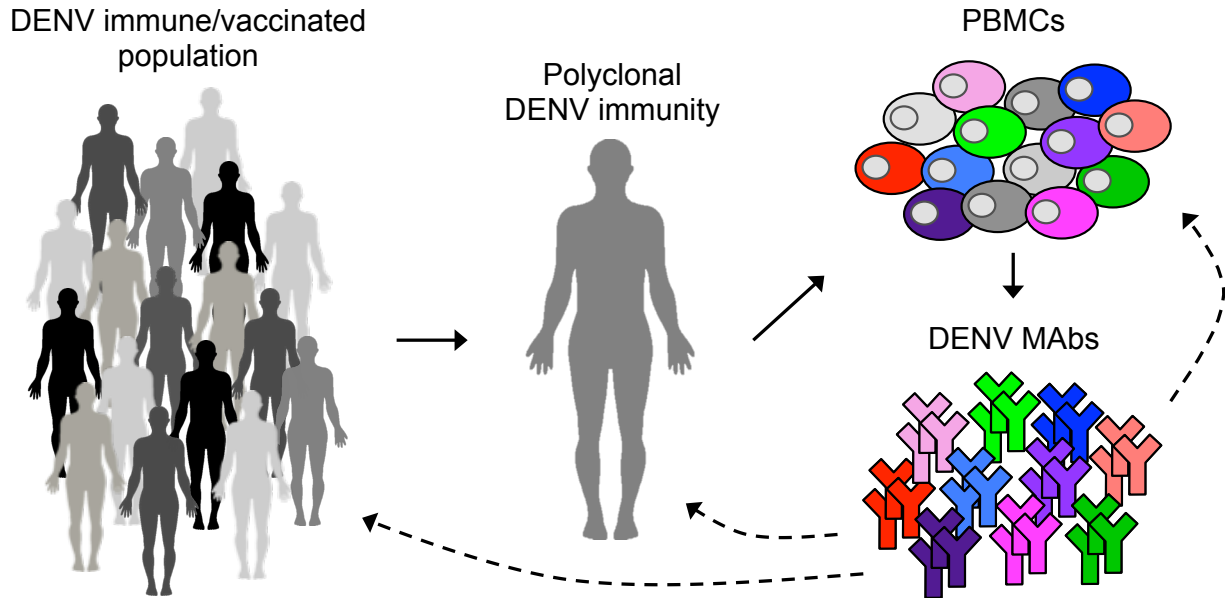
**Figure 1.3. Methods to dissect DENV antibody response.** **A)** Human DENV antibodies can be studied using a variety of approaches. PBMCs from a DENV immune donor can be EBV-transformed to generate MAb producing hybridomas, or antibody DNA sequences can be single-cell sequenced, cloned and recombinant expressed to generate MAbs. DENV polyclonal immune sera can be depleted of different populations of antibodies using beads coated with

DENV antigens to determine the relative importance and neutralization capacity of these different populations. For example, a DENV2 immune sera containing polyclonal Abs (PABs) can be depleted of all DENV cross-reactive antibodies by incubating with beads adsorbed with DENV1, DENV3, and DENV4 antigen, leaving only DENV2 serotype-specific antibodies remaining (heterotypic depletion). Conversely, all DENV antibodies can be depleted using DENV2 antigen (homotypic antigen). **B)** To map the binding and neutralizing epitopes of these MAbs and PABs, they can be evaluated for their ability to bind recombinant E domain III (rEDIII), recombinant E (rE), whole DENV, and chimeric viruses containing transplanted epitopes of multiple DENV serotypes (e.g. rDENV1/3 contains epitopes from both DENV1 and DENV3). These MAbs and PABs can also be evaluated for their ability to neutralize these DENV and chimeric rDENV.

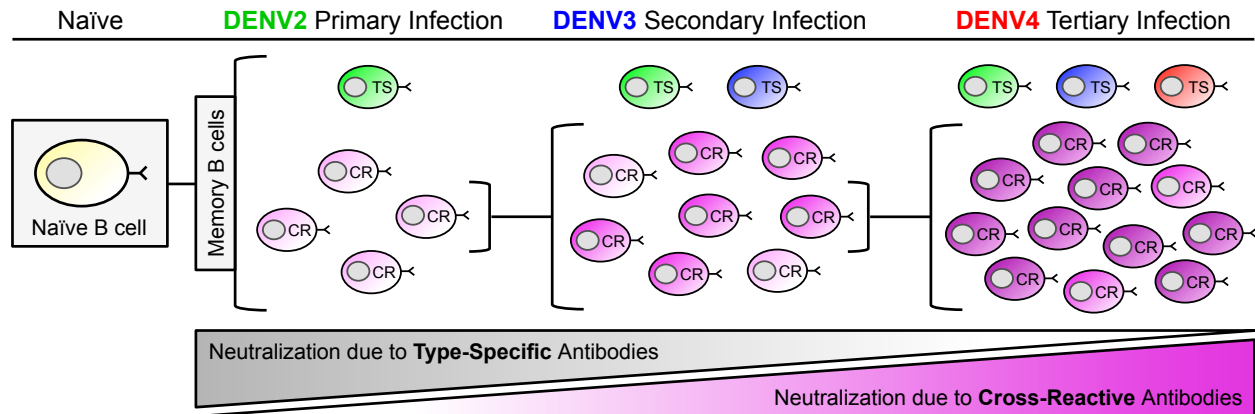


**Figure 1.4. Epitopes recognized by DENV serotype-specific human neutralizing MAbs.** Serotype-specific neutralizing human MAbs isolated from primary infections recognize different quaternary structure epitopes displayed on the viral envelope.



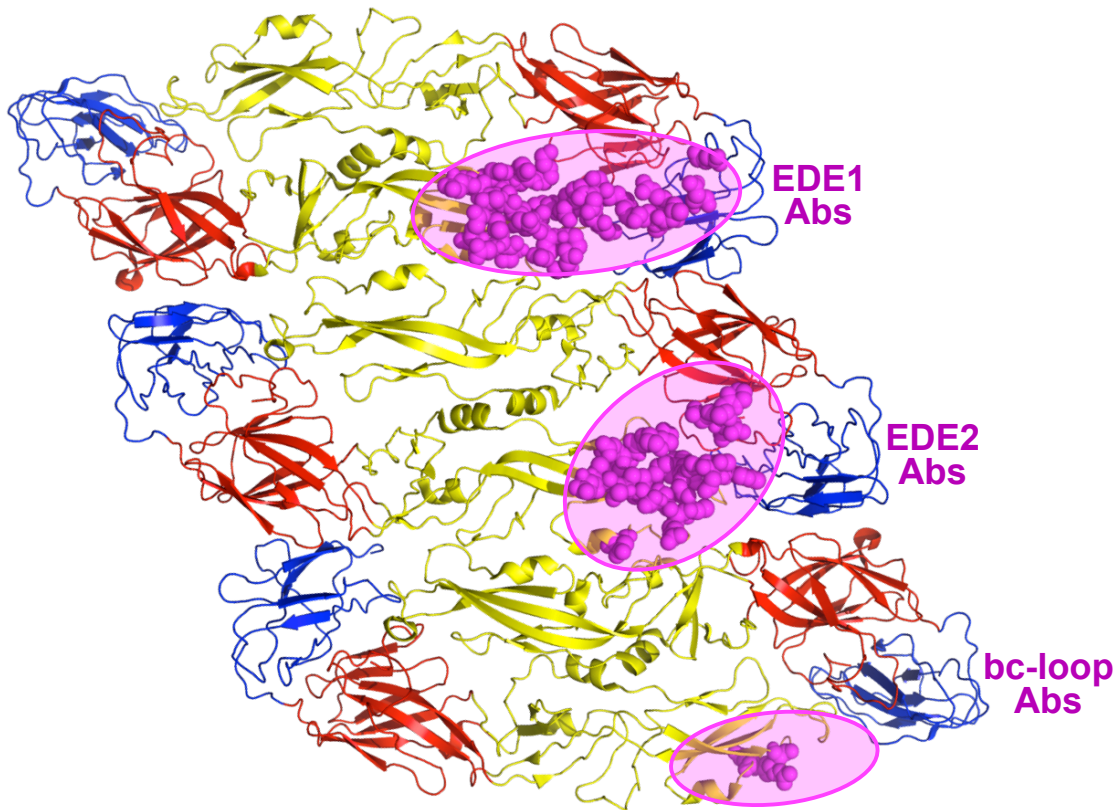


**Figure 1.5. From MAbs to polyclonal serum Abs.** Complex host generic diversity, exposure history, and immune differences can make it challenging to study DENV polyclonal antibody responses across a population. Studying DENV antibody immunity in a single individual can simplify these analyses, however there is still the polyclonal nature of the adaptive immune response. Conversely, we can characterize the properties of individual MAbs from DENV immune donors. Information learned from MAbs can then be used to inform study of the B-cell repertoire from that, and other donors. Additionally, it can be determined whether the individual MAbs represent the polyclonal antibodies in that donor, and in a larger DENV immune population.



**Figure 1.6. Model of B-cell maturation following sequential DENV infections.** With each successive DENV infection, the ratio of serotype-specific (TS) and cross-reactive (CR) antibodies that contribute to DENV neutralization changes. During a primary infection (DENV2 in this example), dengue-specific naïve B-cells are activated and these cells give rise to both memory B-cells (MBCs) and antibody secreting long lived plasma cells (LLPCs). This primary response is dominated by MBCs and LLPCs clones producing low affinity, weakly neutralizing serotype CR antibodies. The primary response also contains rare MBC and LLPCs producing TS antibodies that strongly neutralize DENV2. Following a secondary infection with a new serotype (DENV3 in this example), the overall DENV-specific B-cell response will be dominated by the activation and expansion of DENV2 and 3 cross-reactive MBCs induced by the primary infection. MBCs producing CR antibodies that bind to the second infecting serotype with high affinity will be preferentially activated. These activated cells will reenter germinal centers and undergo further rounds of somatic hyper mutation. CR B-cells with high affinity for the second serotype will be selectively expanded to give rise to cross-reactive MBC and LLPCs that strongly cross-neutralize multiple serotypes. In the figure this increase in affinity and neutralization is depicted by an increase in the color gradient (light pink to bright pink) of CR B-cells. Following a tertiary infection (DENV4 in this example), this process is repeated again and results in a population of CR MBCs and LLPCs that dominate the neutralizing antibody

response. While the B-cell clones producing TS strongly neutralizing antibodies are also likely to be maintained through each successive round of infection, the TS response will account for only a small fraction of the total neutralizing response.



**Figure 1.7. EDE and other cross-reactive epitopes.** Envelope dimer epitope 1 (EDE1) targets EDIII of one monomer and spans over the fusion loop region of EDII of the neighboring monomer. EDE2 uses a similar epitope, but is shifted to also expand into EDI of the first monomer. Another class of cross-reactive antibodies targets the highly conserved bc-loop region of EDII.

## **CHAPTER 2 – A new quaternary structure epitope on dengue virus serotype 2 is the target of durable type-specific neutralizing antibodies**

### **2.1 Summary**

Dengue virus serotype 2 (DENV2) is widespread and responsible for severe epidemics. While primary DENV2 infections stimulate serotype-specific protective responses, a leading vaccine failed to induce a similar protective response. Using human monoclonal antibodies (hMAbs) isolated from dengue cases and structure-guided design of a chimeric DENV, here we describe the major site on the DENV2 envelope (E) protein targeted by neutralizing antibodies. DENV2-specific neutralizing hMAb 2D22 binds to a quaternary structure epitope. We engineered and recovered a recombinant DENV4 that displayed the 2D22 epitope. DENV2 neutralizing antibodies in people exposed to infection or a live vaccine tracked with the 2D22 epitope on the DENV4/2 chimera. The chimera remained sensitive to DENV4 antibodies, indicating that the major neutralizing epitopes on DENV2 and -4 are at different sites. The ability to transplant a complex epitope between DENV serotypes demonstrates a hitherto underappreciated structural flexibility in flaviviruses, which could be harnessed to develop new vaccines and diagnostics.

### **2.2 Importance**

Dengue virus causes fever and dengue hemorrhagic fever. Dengue serotype 2 (DENV2) is widespread and frequently responsible for severe epidemics. Natural DENV2 infections stimulate serotype-specific neutralizing antibodies, but a leading DENV vaccine did not induce a

similar protective response. While groups have identified epitopes of single monoclonal antibodies (MAbs), the molecular basis of DENV2 neutralization by polyclonal human immune sera is unknown. Using a recombinant DENV displaying serotype 2 epitopes, here we map the main target of DENV2 polyclonal neutralizing antibodies induced by natural infection and a live DENV2 vaccine candidate. Proper display of the epitope required the assembly of viral envelope proteins into higher-order structures present on intact virions. Despite the complexity of the epitope, it was possible to transplant the epitope between DENV serotypes. Our findings have immediate implications for evaluating dengue vaccines in the pipeline as well as designing next-generation vaccines.

## **2.3 Introduction**

Dengue virus (DENV) is the most significant arboviral infection of humans, with an estimated 390 million infections and 96 million symptomatic cases annually (1). The DENV complex consists of four distinct serotypes (DENV1-4). Infection with one serotype induces long-term protective immunity to the homologous serotype only. In fact, immunity to one serotype is associated with an increased risk of severe disease upon subsequent infection with a different serotype, a confounding factor for vaccine design. Many dengue vaccines in clinical trials are tetravalent live-attenuated virus formulations that are designed to simultaneously induce protective immunity to all 4 serotypes (71-73). However, in phase 3 efficacy trials in Asia and Latin America, the leading vaccine was 50-78% efficacious against serotypes 1, 3 and 4 but only 35-42% efficacious against serotype 2 (74, 75). Here we describe the main site on DENV2 recognized by type-specific and durable neutralizing antibodies in people exposed to natural infections, after administration of a candidate vaccine in humans and in macaques infected with wild-type dengue virus strains.

The DENV envelope glycoprotein (E) is the main target of protective antibodies (5). The E protein is composed of three domains: I, II and III (designated EDI, EDII and EDIII). Each DENV particle has 180 monomers of E that are organized into 90 dimers that cover the entire surface of the virus (76). The arrays of E proteins are arranged with icosahedral symmetry, with each asymmetric unit containing three E proteins. Some human monoclonal antibodies (hMAbs) that neutralize DENVs bind to quaternary structure epitopes that require assembly of E protein into homodimers or higher order structures (27, 36, 38, 51, 77). Following infection or vaccination, it is a DENV-specific serum polyclonal antibody response that is responsible for protection. The principle targets of the human polyclonal antibody responses that neutralize DENVs have remained elusive.

We recently described hMAb 2D22, which is a DENV2-specific strongly neutralizing antibody isolated from a person exposed to a primary DENV2 infection (27). A point mutation at amino acid position 323 in EDIII (residue highlighted in magenta in Figure 2.1A and B) led to complete escape from 2D22 neutralization, indicating that the epitope includes EDIII residues (27). Recently Fibriansah et al solved the structure of 2D22 bound to DENV2 and demonstrates that the antibody bound to a quaternary epitope that was formed by EDIII and EDII on two different monomers within a single dimer (38). While the structure of hMAb 2D22 was a major advance, it is not known if the structurally intriguing epitope defined by 2D22 is the main target of neutralizing and protective antibodies in people exposed to DENV2 infections or a vaccine.

## **2.3 Results**

### *Design of recombinant DENV4/2 chimeric virus*

To further understand the role of EDIII in the epitope of 2D22 and DENV2 neutralizing antibodies in general, we designed and recovered a recombinant chimeric virus in which the entire DENV2 EDIII region was inserted into the backbone sequence of a DENV4 molecular

clone to create a recombinant virus, designated rDENV4/2 (Figure 2.2). The recombinant virus, which had 40 amino acid changes in EDIII compared to the parental wild-type (*wt*) DENV4 strain (Figure 2.1A and Table 2.1), grew to similar levels as the *wt* viruses in C6/36 insect cells and in a human monocytic cell line (U937) expressing dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), a known dengue receptor, but was partially growth impaired in Vero cells (Figure 2.1C). DENVs are assembled inside cells as immature virions containing premembrane proteins (prM), which are cleaved in the Golgi resulting in the formation of mature virions. Proteolytic cleavage of prM is inefficient and the population of virions released from infected cells is an admixture displaying different stages of maturation. As the maturation state of DENVs may influence the display of some epitopes and sensitivity to antibody neutralization (59, 62, 78), immunoblots were performed to compare the maturation state of the rDENV4/2 chimera and the *wt* parental strains. From C6/36 cells, DENV2 particles had high levels of prM and DENV4 particles had low levels of prM relative to E protein, indicating that DENV4 virions were more mature (Figure 2.1D). The rDENV4/2 chimera had a maturation state similar to DENV4 indicating that insertion of EDIII from serotype 2 minimally altered the maturation state of the backbone serotype 4 virus (Figure 2.1D).

#### *Monoclonal antibody binding and neutralization of rDENV4/2*

To further evaluate the impact of EDIII exchange on overall E protein topology and virion structure, we probed the rDENV4/2 chimera with a panel of epitope-mapped human and mouse monoclonal antibodies (Table 2.2). DENV cross-reactive MAbs 1C19, 1N5 and 1M7 (50) bound to the chimera, indicating the preservation of cross-reactive epitopes (Figure 2.3A). DVC3.7 and DV4-E88 engage serotype-specific epitopes on EDIII of DENV2 and 4 respectively (Figure 2.3B, C and Table 2) (26, 79). Consonant with recombinant virus design, DVC3.7 bound and neutralized the chimera, whereas DV4-E88 failed to bind or neutralize the chimera (Figure 2.3F and G). Binding to and neutralization by 5H2, a non-human primate DENV4 serotype-specific



MAB with an EDI epitope, is not disrupted in rDENV4/2, showing we have not affected epitopes present on other domains (Figure 3.2D, H and Table 2.2) (80). Overall, these results demonstrate that the rDENV4/2 chimera displays epitopes in a manner consistent with display on a properly folded and functional chimeric E protein. Transplantation of DENV2 EDIII into DENV4 also restored binding and neutralization by MAb 2D22, even though this antibody did not bind to DENV2 recombinant EDIII alone (Figure 2.3E, I and Table 2.2). We predict that the full 2D22 epitope required for antibody binding and neutralization includes EDIII as well as some conserved residues on adjacent domains, but that the residues on EDIII alone determine DENV2 specificity. Indeed the cryo-electron microscopy structure of 2D22 bound to DENV2 demonstrates that the epitope consists of residues on EDIII and EDII of different monomers within a single dimer (38).

#### *Polyclonal sera neutralization of rDENV4/2*

Natural primary DENV2 infections cause long-lived serotype-specific neutralizing antibody responses that can be detected for decades after exposure. To determine if “2D22-like” epitopes created by DENV2 EDIII transplantation into DENV4 were the main targets of these antibodies, neutralization assays were performed with well characterized human and rhesus macaque dengue immune sera (Table 2.3) and the rDENV4/2 and parental viruses. As expected, primary DENV2 immune sera strongly neutralized DENV2, but not DENV4 (Figure 2.4A). Remarkably, in the majority of cases, these sera also efficiently neutralized the rDENV4/2 virus at levels similar to those measured with DENV2, indicating that EDIII replacement was sufficient to recreate the major DENV2 neutralizing epitopes recognized by these sera. The rDENV4/2 virus remained fully sensitive to neutralization of DENV4 immune sera (Figure 2.4B). These data suggest that DENV2 and DENV4 type-specific neutralizing antibodies target different epitopes on the E protein, which are both preserved on the rDENV4/2 chimera. To determine if neutralization of rDENV4/2 is specific to DENV2 and DENV4 immune sera, and not

reflecting a global increase in sensitivity to neutralization by any dengue immune serum, a panel of primary DENV1 and DENV3 immune sera were tested against the same three viruses. The rDENV4/2 virus did not display increased sensitivity to neutralization by DENV1 or 3 immune sera ( $p>0.05$ ;  $p>0.05$ ), demonstrating that the chimeric virus was not globally sensitive to antibody neutralization (Figure 2.4C and D).

#### *DENV2 type-specific antibodies require complex epitope*

To determine if the DENV2 neutralizing epitope recognized by antibodies in immune sera was entirely contained within the transplanted EDIII or included residues on EDIII and adjacent domains, the human immune sera were depleted of antibodies binding intact DENV2 virions or recombinant DENV2 EDIII (Figure 2.5) and then tested for ability to neutralize the rDENV4/2 virus (Figure 2.6). We have previously demonstrated that the recombinant DENV2 EDIII protein is properly folded and contains well-defined epitopes such as the lateral ridge and A-strand epitopes recognized by some mouse and human neutralizing antibodies (28, 81). When six primary DENV2 human immune sera were depleted using DENV2 virions, between one half to three quarters of the neutralizing potency was lost depending on the serum sample (Table 2.4) ( $68\% \pm 21\%$ ). When the same sera were depleted using DENV2 rEDIII alone, five sera displayed minor loss in neutralization ( $22\% \pm 3\%$ ) and one sample (DT110) lost 58% of neutralization, a significantly smaller loss of neutralization than compared to DENV2 depletions (Table 2.4) ( $p<0.05$ ). These results indicate that most DENV2 epitopes targeted by polyclonal type-specific human neutralizing antibodies require assembly of more higher order structures than simple domains or monomers of E protein. We conclude that “2D22-like” EDIII-containing quaternary epitopes are a major target of serotype 2-specific long-lived polyclonal neutralizing antibodies that develop after DENV2 infections.

To determine if dengue vaccines can induce “2D22-like” quaternary epitope targeted neutralizing antibodies, we tested sera from 5 subjects who had developed DENV2 neutralizing antibodies after receiving a monovalent live attenuated DENV2 vaccine developed by the NIH (82). The vaccine sera neutralized DENV2 and the rDENV4/2 chimera but not DENV4 demonstrating that the vaccine induced neutralizing antibodies that tracked with the transplanted EDIII (Figure 2.4) ( $p < 0.01$ ). To determine if the vaccine induced antibodies also recognized a quaternary epitope that extended beyond EDIII, three vaccine sera were depleted of antibodies binding intact DENV2 virions or recombinant DENV2 EDIII and then tested for ability to neutralize the chimeric virus. In all three samples depletion with whole virus led to a nearly complete loss of neutralizing antibodies (Table 2.4). Removal of EDIII specific antibodies resulted in a loss of neutralizing antibodies in one vaccine sample, while the other two samples retained the majority of neutralizing antibodies after EDIII depletion (Table 2.4). Thus, the vaccine induced neutralizing antibodies that bind to epitopes contained within EDIII or more complex epitopes that extend beyond EDIII.

## **2.5 Discussion**

We have described an approach using whole domain replacement to identify principal antigenic sites targeted by polyclonal antibodies following natural DENV infection or experimental live attenuated DENV vaccination. With the DENV4/2 chimera, we observed a clear gain of DENV2 neutralization and no loss of sensitivity to neutralization by DENV4 sera, suggesting that the principal DENV4 neutralizing epitopes are distinct from DENV2 epitopes. Importantly, these data demonstrate that a single recombinant DENV can be designed that encodes major neutralizing epitopes from two virus serotypes.

Several recent studies point to the importance of quaternary epitopes as targets of human DENV neutralizing antibodies (27, 36, 38, 51, 77). DENV1 and 3 neutralizing hMAbs

recognize distinct quaternary structure epitopes centered at the EDI/II hinge. However, only a small fraction (<3%) DENV-specific memory B-cell clones produce strongly neutralizing antibodies (83). It has not been clear if epitopes defined using human MAbs are the main targets of the polyclonal serum neutralizing antibody response as well. Our studies here demonstrate that the DENV2 serotype-specific epitopes targeted by a human MAb, and polyclonal immune sera are closely related if not identical. The epitope is a complex, quaternary epitope and includes critical residues in EDIII that determine serotype specificity.

The results reveal the fundamental importance of complex quaternary structures on the surface of DENV particles for driving potent antibody immune responses. Our results are entirely consistent with the 2D22 epitope structure reported by Fibriansah et al (84) demonstrating that antibody footprint contains critical contact residues on EDIII of one monomer, as well as the fusion loop and BC-loop of EDII on the adjacent monomer, bridging across the dimer. The structure also demonstrates that the 2D22 antibody contact sites on EDIII are not conserved between serotypes but the contact sites on EDII are highly conserved between DENV2 and 4 (84). Thus, the serotype specificity of the 2D22 is determined by EDIII and transplantation of this domain into DENV4 was sufficient to create the complete epitope, functional epitope.

Dengue vaccines have been challenging to develop because of the need to formulate vaccines with four components that simultaneously induce durable neutralizing and protective antibodies to each serotype. Without knowing the identity of critical epitopes and regions on viruses of the four serotypes targeted by neutralizing and protective polyclonal serum antibodies, it has been difficult to dissect tetravalent vaccine responses and efficacy data from ongoing clinical trials. Our results demonstrate that the NIH monovalent live attenuated DENV2 vaccine induces neutralizing antibodies that are similar to those induced by natural infection.

The rDENV4/2 chimera is a powerful tool for evaluating antibody site-specific responses following infection and vaccine trials. Recombinant DENVs expressing quaternary neutralizing antibody epitopes from 2 or more serotypes may lead to simpler and more effective vaccines than current tetravalent formulations.

## **2.6 Materials and Methods**

### *Virus construction*

Recombinant viruses were constructed using a four-cDNA cloning strategy, the same strategy used to create *wt* DENV infectious clones (Figure 2.2). Patterned after coronavirus cDNA clones (85, 86), the DENV-4 genome was subcloned into four separate cDNA plasmids. A T7 promoter was introduced into the 5' end of the A fragment, and unique type IIS restriction endonuclease cleavage sites are introduced into the 5' and 3' end of each fragment to allow for systematic assembly into a genome-length cDNA from which full-length transcripts can be derived (85-87).

The EDIII residues from DENV2 were introduced into the DENV4 A subclone by replacing E nucleotides 900-1179 with the corresponding nucleotides encoding variant DENV2 amino acids. The new A fragment with nucleotides from DENV2 was synthesized and inserted into pUC-57 plasmid (BioBasic). The new A plasmid and the DENV4 B, C and D plasmids were grown in *E. coli*, purified, digested with corresponding type IIS restriction enzymes, and ligated using T4 DNA ligase to create a full-length cDNA dengue viral genome. The full-length cDNA was transcribed into genome-length RNAs using T7 polymerase, as previously described by our group (85-87). Recombinant RNA was electroporated into BHK-21 cells and cell culture supernatant containing viable virus was harvested. Virus was then passaged two times on

C6/36 cells, centrifuged to removed cellular debris, and stored at -80°C. Passage 3 represents our working stock.

### *Cells*

Mosquito *Ae. albopictus* C6/36 cells were grown in MEM (Gibco) media at 32°C. Vero-81 cells were maintained in DMEM and U937+DC-SIGN were maintained in RPMI at 37°C. Medium was supplemented with FBS (10% for Vero-81 and 5% for C6/36 and U937+DC-SIGN), which was lowered to 2% after infection. C6/36 and U937+DC-SIGN media was supplemented with non-essential amino acids, and U937+DC-SIGN was also supplemented with L-glutamine and 2-mercaptoethanol. All media were additionally supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. All cells were incubated in 5% CO<sub>2</sub> as previously described by our group (87).

### *DENV Type-Specific PCR and RFLP Analysis*

Total RNA was isolated from viral supernatants and used as template for cDNA synthesis using standard molecular techniques. Serotype-specific PCR and restriction endonuclease analyses were performed on cDNA samples in order to validate purity of the recombinant viral preparations (Figure 2.7).

### *Binding ELISA*

Equal quantities of virus (as previously titrated by ELISA) were captured using either mouse anti-DENV MAbs 4G2 and 2H2, or human MAb 1C19. Primary antibodies were diluted four-fold starting at concentrations varying from 10 ng/µL-100 ng/µL. Alkaline phosphatase-conjugated secondary antibodies were used to detect binding of primary antibodies with P-nitrophenyl phosphate substrate, and reaction color changes were quantified using spectrophotometry, as previously described (26).

### *DENV Immune Sera*

De-identified human DENV immune sera were collected from individuals with confirmed previous natural DENV infections (Table 2.3). All donations were collected in compliance with the Institutional Review Board of the University of North Carolina at Chapel Hill (Protocol #08-0895). De-identified human immune sera previously collected from adults given the NIH monovalent DENV2 vaccine (ClinicalTrials.gov identifier: NCT00920517) was provided by Anna Durbin and Stephen Whitehead. All sera was collected following informed consent and approval by the Western Institutional Review Board. Non-human primate immune sera were collected following experimental DENV infection, and kindly provided by Carlos Sariol (Supplemental Table 2). All procedures were reviewed and approved by the Institute's Animal Care and Use Committee at Medical Sciences Campus, University of Puerto Rico (IACUC-UPR-MSC), and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) (Animal Welfare Assurance number A3421; protocol numbers, 7890108, 7890208, 7890209, and 7890210).

### *Virus Titration and Focus Reduction Neutralization Test (FRNT)*

One day prior to inoculation, 24-well cell culture plates were seeded with either  $5 \times 10^4$  Vero-81 or  $1 \times 10^5$  C6/36 cells. Prior to inoculation, growth medium was removed. Virus titrations were performed by serially diluting virus stocks 10-fold, then incubated for 1 hr at 37°C. After incubation, virus dilutions were added to cells for 1 hr at 37°C, then overlaid with 1 mL 1% methylcellulose in OptiMEM I (Gibco), supplemented with 2% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. After 3-6 days incubation at 37°C, overlay was removed, and cells were washed with PBS and fixed in 80% methanol. Plates were blocked with 5% instant milk made in PBS, then incubated with anti-E MAb 4G2 and anti-prM MAb 2H2, both diluted 1:500 in blocking buffer. Plates then were washed and incubated with HRP-conjugated goat anti-mouse Ab

(Sigma), diluted 1:2,500 in blocking buffer. Plates were washed, foci were developed with TrueBlue HRP substrate (KPL), and then foci were counted.

For the FRNT assay, either MABs or sera were diluted four-fold and mixed with ~40 focus forming units (FFUs) virus, then incubated for 1 hr at 37°C. After incubation, virus and MAb or serum dilutions were added to cells for 1 hr at 37°C, then overlay was added and processed as above.

### *Growth Curves*

Either Vero or C6/36 cells were inoculated at a multiplicity of infection (MOI) of 0.01. Every 24 hrs, culture supernatant was harvested and centrifuged to remove cellular debris. Samples were frozen at -80°C until use. Fresh medium was replaced each day. Viruses were titrated on their propagating cell type, as described above. U937+DC-SIGN cells were infected at an initial infection of 1%, and every 12 hours a sample of cells was harvested, fixed, permeabilized, and probed with 2H2 (anti-prM antibody) conjugated to 488. Infected cells were quantified using a Guava flow-cytometer (Milipore).

### *Immunoblotting*

Virus stocks were diluted in PBS, mixed with 4X Laemmli Sample Buffer (Bio-Rad), and heated for 10 minutes at 50°C. Samples were run on 12% PROTEAN TGX Gels (Bio-Rad), transferred to PVDF membrane and blocked in 5% instant milk in PBS + 0.05% Tween overnight at 4°C. Membranes were probed with 0.5 µg/mL anti-E MAb 4G2, 0.5 µg/mL anti-prM MAb 2H12 and MAb 5L20 in blocking buffer for 2 hr at 37°C. After washing, HRP-conjugated anti-mouse or anti-human secondary antibodies were diluted 1:10,000 in blocking buffer, and incubated 1 hr at room temperature. Membrane was exposed to chemiluminescent substrate and developed on film.



#### *Depletion of DENV2-Specific Antibodies from Immune Sera*

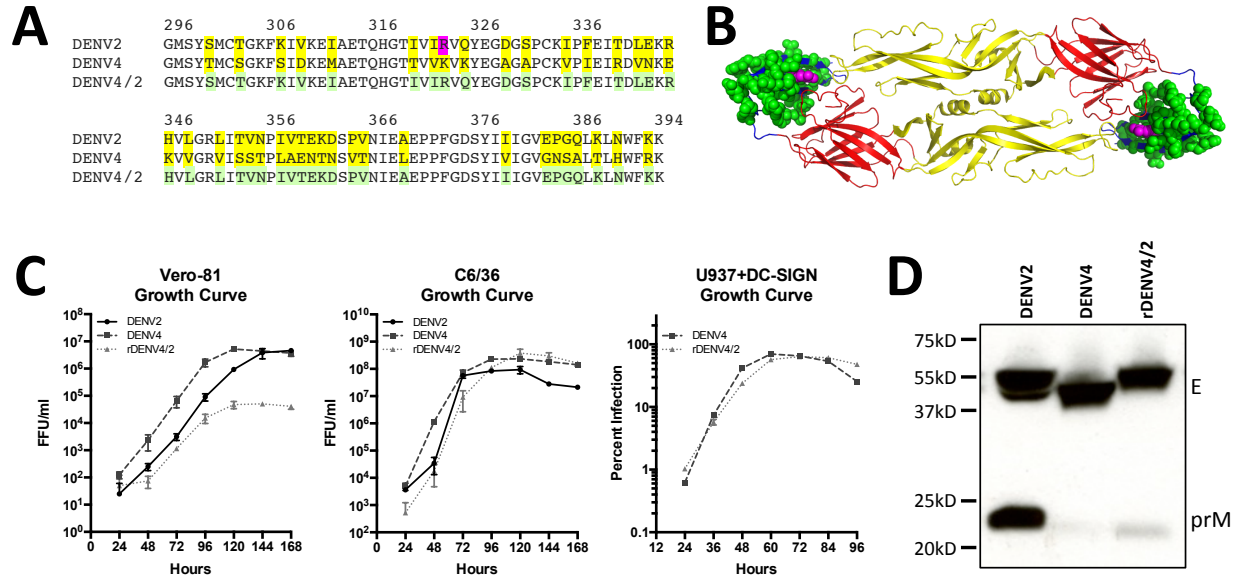
Polyclonal immune sera were depleted of DENV2-binding antibodies as previously described (27). Briefly, polystyrene microspheres (Polysciences, 17135) were coated with purified DENV2 antigen (Microbix, EL-22-02-001) or BSA control. Immune sera were depleted of antibodies by incubating with coated beads for 45 minutes at 37°C for at least three rounds, until maximum depletion of antibodies was measured. Depletions of antibodies were confirmed by ELISA.

#### *Depletion of rEDIII-Specific Antibodies from Immune Sera*

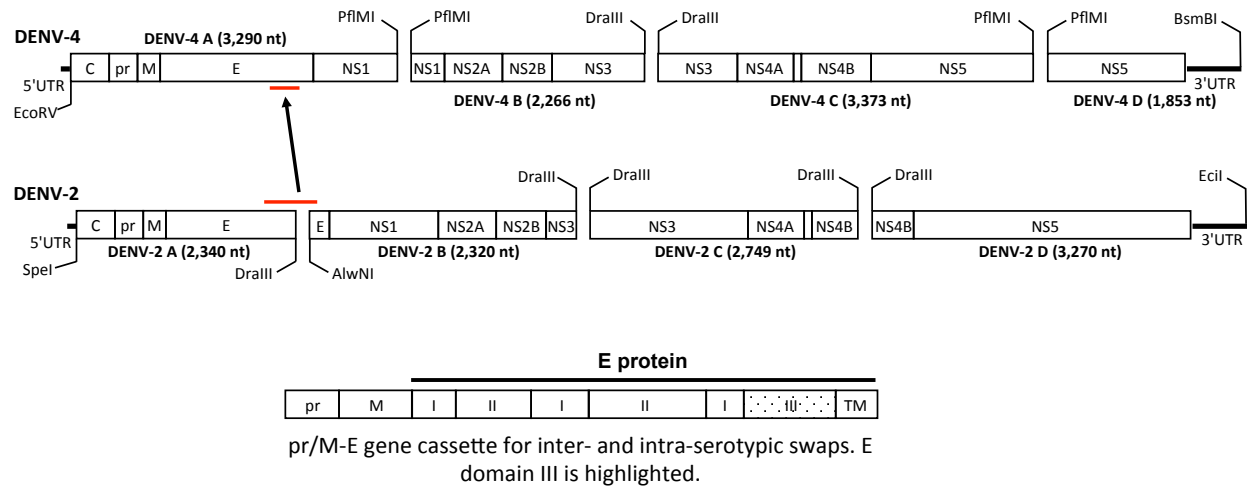
Polyclonal immune sera were depleted of rEDIII-binding antibodies as previously described for rE-binding antibodies (27). Briefly, Dynabeads® (Life Technologies, 14302D) were covalently conjugated to DENV2 rEDIII protein following manufacturers protocol or BSA control. Immune sera were depleted of antibodies by incubating with conjugated beads for 45 minutes at 37°C for at least three rounds, until maximum depletion of antibodies was measured. Depletions of antibodies were confirmed by ELISA.

#### *ELISA Confirmation of DENV2 or rEDIII Depleted Sera*

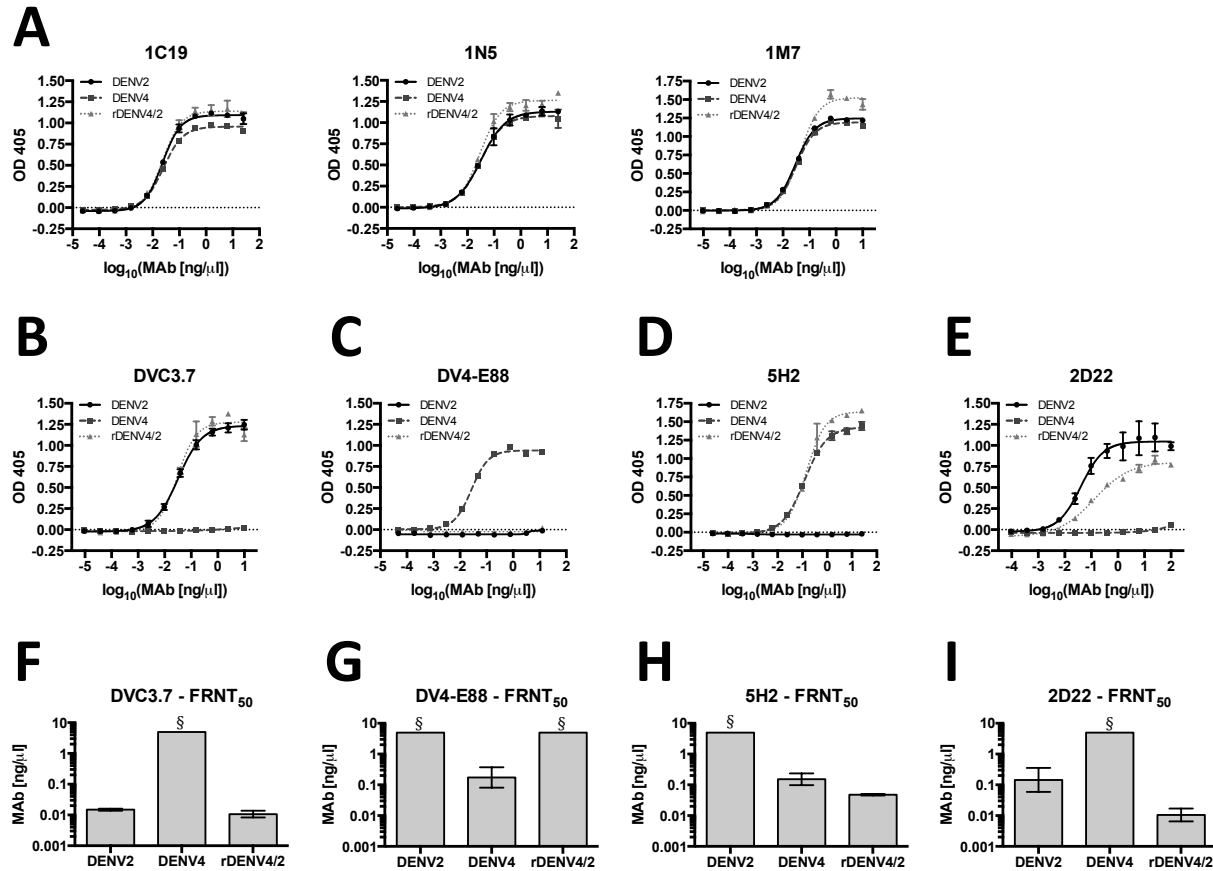
ELISA plates were coated directly with either 50ng of DENV2 antigen or 100ng DENV2 rEDIII per well at 4°C overnight. Plates were blocked as described above. Undepleted, control-depleted and antigen/rEDIII-depleted sera were diluted 1:40 in blocking buffer and were incubated on plates for 1 hour at 37°C. DENV2 or rEDIII reactive antibodies were detected using secondary antibody and substrate as described above.



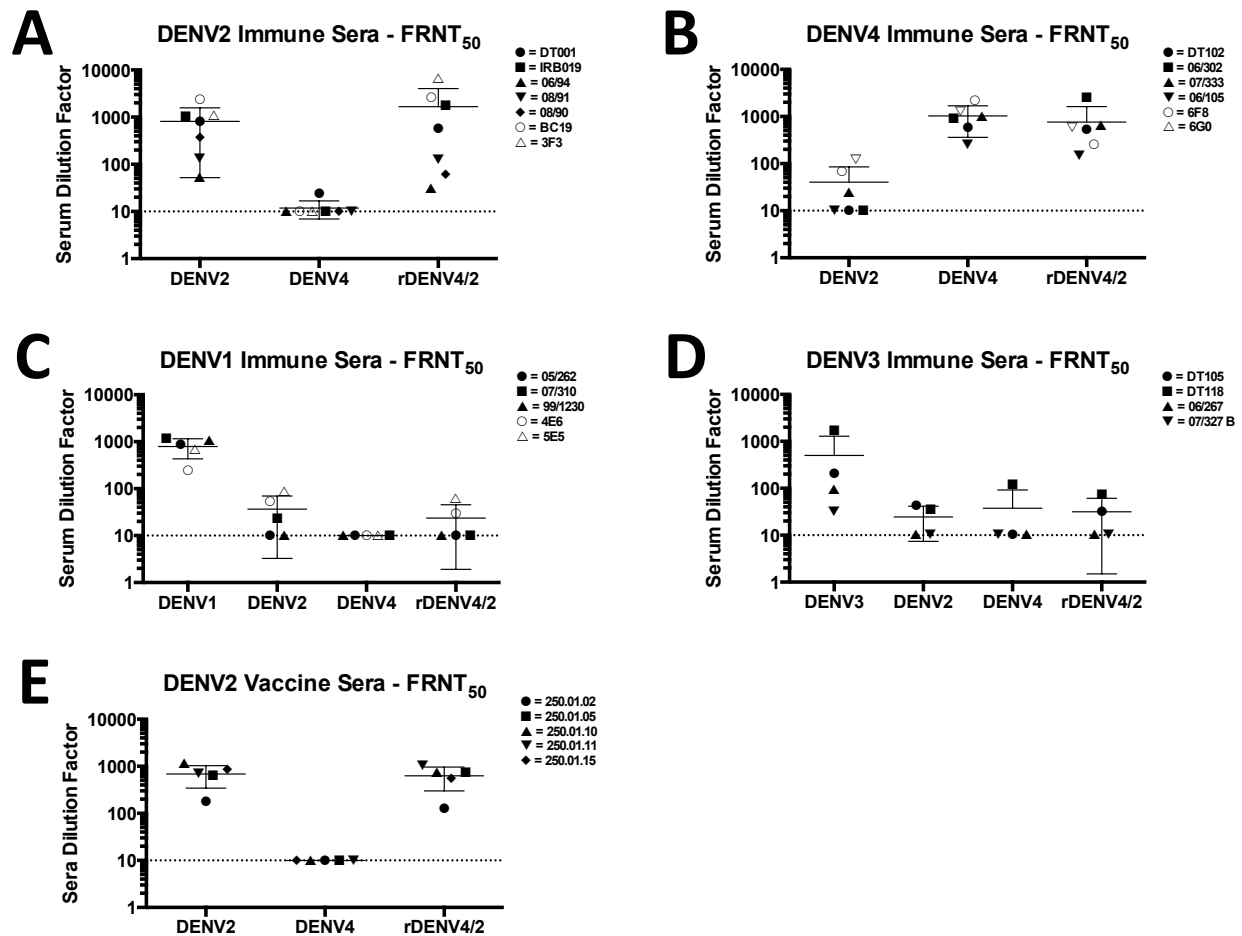
**Figure 2.1. Design and characterization of rDENV4/2.** (A) Amino acid alignment of DENV2 and DENV4 linear envelope domain III (EDIII) sequence, residues 296-395 of entire E sequence (99 aa total). Residues differing between DENV2 and DENV4 are highlighted in yellow. Recombinant DENV4 virus containing EDIII from DENV2, designated rDENV4/2, replaces differing residues from DENV4 with those from DENV2, highlighted in green (40 aa total). Residue generated from escape mutant highlighted in magenta. (B) Crystal structure model of DENV2 E protein dimer, with swapped residues colored in green, and DENV2 type-specific MAb 2D22 escape mutant residue highlighted in magenta. (C) Vero-81 or C6/36 cells were inoculated and viral supernatants were collected every 24 hrs and subsequently titrated on the respective cell type, or 1% of U937+DC-SIGN were infected and total percent infection was measured every 12 hours (mean  $\pm$  s.d.) (D) Immunoblotting of C6/36 grown viruses with anti-E and anti-PrM antibodies. E = 55 kD, prM = 21 kD.



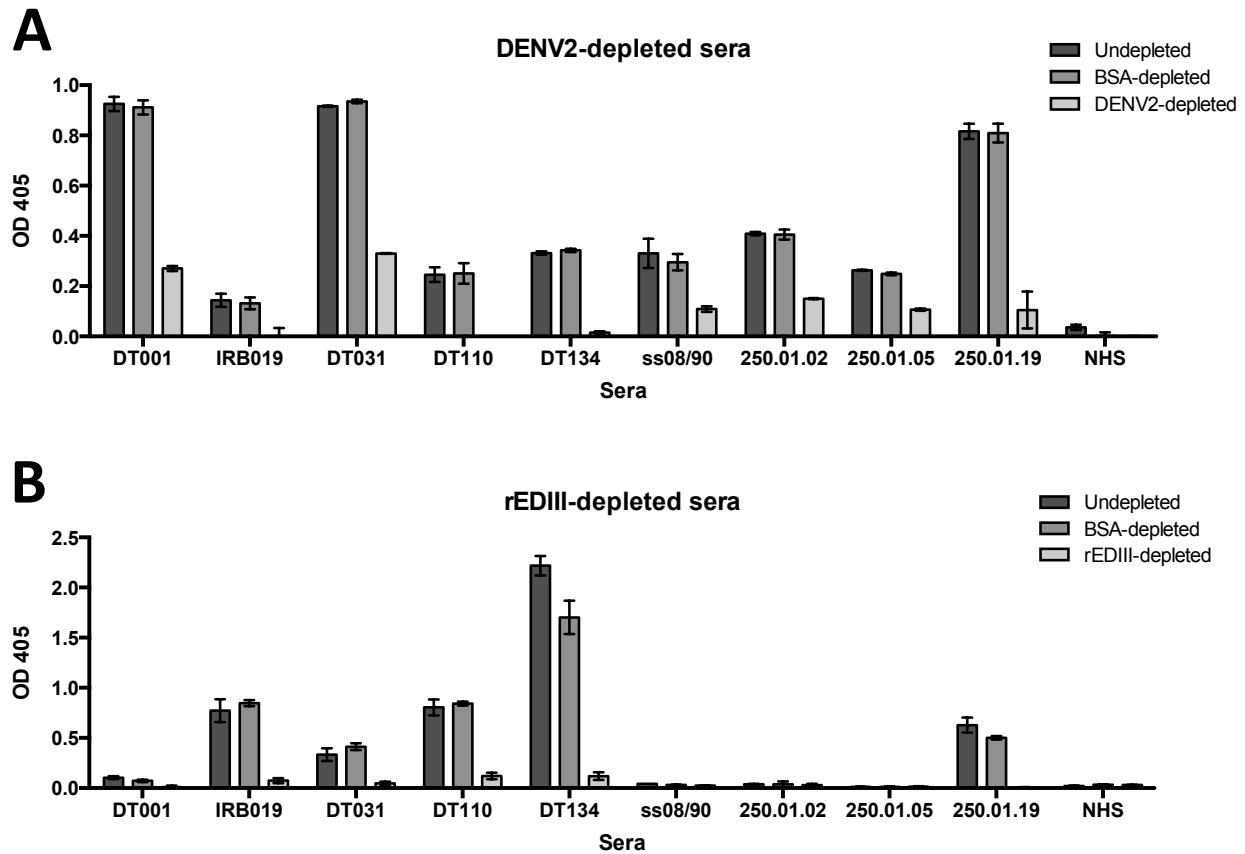
**Figure 2.2. DENV4 infectious clone.** Reverse genetics system for manipulating DENV4 genome. DENV genome was divided into four plasmid cassettes that can be mutated individually, ligated together, and electroporated into cells to generate recombinant virus. DENV4-A cassette contains the envelope gene, EDIII is highlighted grey. Replacing EDIII residues with those from DENV2, in DENV4 backbone, creates rDENV4/2 recombinant virus. A total of 58 nucleotide changes were introduced into DENV4-A cassette (highlighted in red).



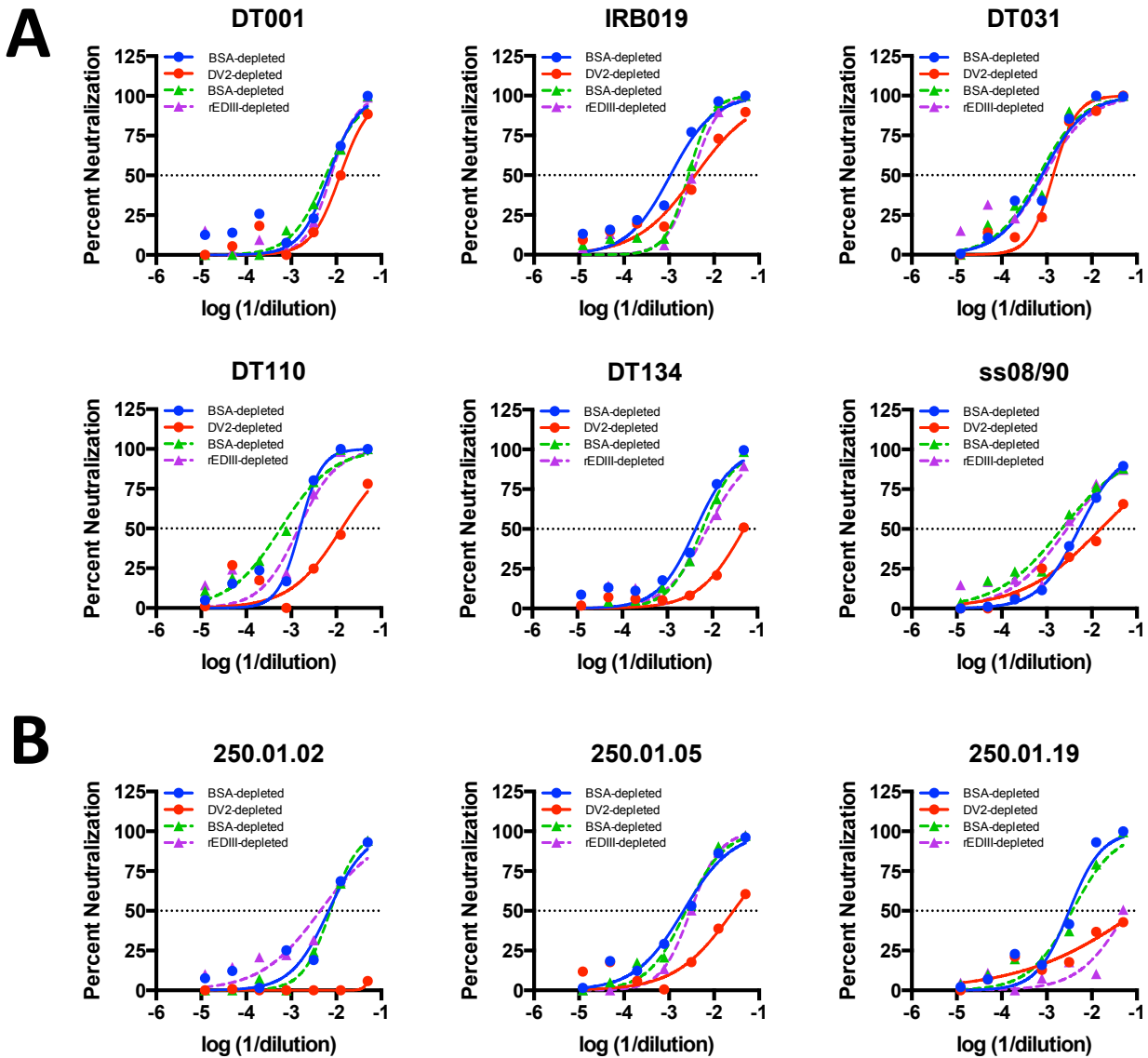
**Figure 2.3. Recognition and neutralization of rDENV4/2 virus by DENV-specific monoclonal antibodies.** ELISA capture assay with (A) cross-reactive MABs, (B) DENV2-specific EDIII MAB DVC3.7, (C) DENV4-specific EDIII MAB DV4-E88, (D) DENV4-specific EDI MAB 5H2, and (E) DENV2-specific MAB 2D22 (mean  $\pm$  s.d.). Vero-81 cell based Focus Reduction Neutralization Test (FRNT) was performed using (F) DENV2-specific EDIII MAB DVC3.7, (G) DENV4-specific EDIII MAB DV4-E88, (H) DENV4-specific EDI MAB 5H2, or (I) DENV2-specific MAB 2D22 and FRNT<sub>50</sub> (concentration of antibody required to neutralize 50% of infection) values were calculated (mean  $\pm$  95% CI), § = FRNT<sub>50</sub> >5 ng/μl



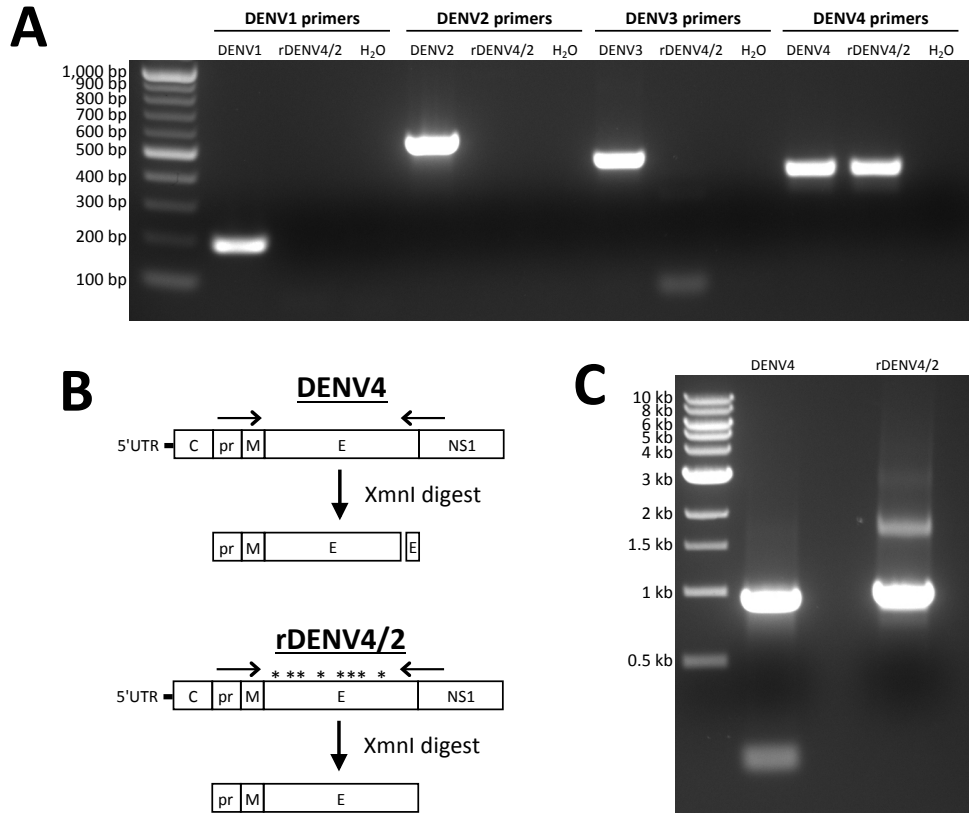
**Figure 2.4. rDENV4/2 neutralization by human and macaque DENV immune sera.** Vero-81 cell-based Focus Reduction Neutralization Test (FRNT) was performed using (A) primary DENV2, (B) primary DENV4, (C) primary DENV1, (D) primary DENV3, and (E) monovalent DENV2 vaccine immune sera, and FRNT<sub>50</sub> (sera dilution factor required to neutralize 50% of infection) values were calculated (mean  $\pm$  95% CI). Solid symbols = human sera, empty symbols = rhesus macaque sera. Sera that did not block 50% of infection at lowest sera dilution factor were assigned a value of 10 ( $\frac{1}{2}$  the lower limit of detection) for graphing and statistical analysis.



**Figure 2.5. Depletion of DENV2- and rEDIII-binding antibodies.** (A) DENV2 immune sera were depleted using beads coated with virus (DV2-depleted) or BSA (BSA-depleted) and removal of DENV2 binding antibodies was confirmed by ELISA, with wells directly coated with DENV2 antigen. (B) DENV2 immune sera were depleted using Dynabeads with DENV2 rEDIII (rEDIII-depleted) or BSA (BSA-depleted) and removal of DENV2 rEDIII-binding antibodies was confirmed by ELISA, with wells directly coated with rEDIII protein. DT001, IRB019, D031 are sera from people exposed to natural DENV2 infections. 250.01.02, 250.01.05 and 250.01.19 are sera from people who received the NIH DENV2 vaccine. NHS = normal human serum.



**Figure 2.6. rDENV4/2 neutralization by DENV2 and rEDIII depleted sera.** Vero-81 cell-based Focus Reduction Neutralization Test (FRNT) was performed using (A) depleted DENV2 primary immune sera and (B) depleted DENV2 vaccine sera. I = sera depleted using Dynabeads®, ▲ = sera depleted using polystyrene microspheres.



**Figure 2.7. Validation of recombinant virus purity.** (A) Viruses were grown in C6/36 cells, culture supernatant was collected and centrifuged to remove any cellular debris. Viral RNA was isolated using QIAGEN QIAamp Viral RNA Miniprep Kit. Serotype specific PCR was run for 35 cycles, and PCR product was analyzed on 1.5% Ultrapure agarose gel. Control RNA (DENV1/DENV2/DENV3/DENV4) and water were run as positive or negative controls. Expected product sizes: DENV1 = 205 bp, DENV2 = 539 bp, DENV3 = 455 bp, DENV4 = 401 bp. (B) Restriction fragment length polymorphism (RFLP) design to distinguish rDENV4/2 (bottom) from parental DENV4 (top). Mutations (represented as asterisks) introduced into DENV4 E gene to generate rDENV4/2 disrupted XmnI enzyme restriction site present in DENV4. (C) PCR was used to amplify the E gene in both DENV4 and rDENV4/2. PCR products were gel purified and digested with XmnI. Digest products were analyzed on 1.5% Ultrapure agarose gel. Expected product sizes: full length undigested = 1,031 bp, digested products = 931 bp and 113 bp.



Position	DENV2	DENV4	AA change
307	K	S	positive charge → polar uncharged
309	V	D	hydrophobic uncharged → negative charge
320	I	T	hydrophobic uncharged → polar uncharged
325	Q	K	polar uncharged → positive charge
329	D	A	negative charge → hydrophobic uncharged
331	S	A	polar uncharged → hydrophobic uncharged
340	T	R	polar uncharged → positive charge
343	E	N	negative charge → polar uncharged
345	R	E	positive charge → negative charge
353	V	S	hydrophobic uncharged → polar uncharged
358	T	E	polar uncharged → negative charge
359	E	N	negative charge → polar uncharged
360	K	T	positive charge → polar uncharged
361	D	N	negative charge → polar uncharged
364	V	T	hydrophobic uncharged → polar uncharged
382	E	G	negative charge → hydrophobic uncharged
383	P	N	hydrophobic uncharged → polar uncharged
384	G	S	hydrophobic uncharged → polar uncharged
385	Q	A	polar uncharged → hydrophobic uncharged
387	K	T	positive charge → polar uncharged
389	N	H	polar uncharged → positive charge

**Table 2.1. Summary of amino acid changes in rDENV4/2.** Of 40aa residues that were changed in recombinant virus, 14 introduced a change of charge. Six residues increase negative charge (light red = positive → uncharged, dark red = uncharged → negative), while eight residues increase positive charge (light blue = negative → uncharged, dark blue = uncharged → positive). Additional residue changes replace hydrophobic amino acids with polar amino acids, and vice versa.

MAb	Binding			Neutralization	Epitope information
	Whole virus	rE	rEDIII		
2D22	+	-	-	DENV2 only	Quaternary epitope, escape mutant - R323F (EDIII)
DVC3.7	+	+	+	DENV2 only	EDIII lateral ridge
DV4-E88	+	+	+	DENV4 only	EDIII
5H2	N/A	+	N/A	DENV4 only	EDI
1C19	+	+	-	Cross neutralizing	EDII fusion loop and BC loop
1M7	+	+	-	Cross neutralizing	EDII fusion loop region
1N5	+	+	-	Cross neutralizing	EDII fusion loop region

**Table 2.2. Summary of MAbs used in this study.**

	Sera	Species	Date of infection	Location of infection	Time since infection of blood draw
<b>DENV1 immune sera</b>	4E6	<i>R. macaque</i>	5/31/2011	San Juan, Puerto Rico	12 months
	5E5	<i>R. macaque</i>	5/31/2011	San Juan, Puerto Rico	12 months
	05/262	Human	History not known		
	07/310	Human	History not known		
	99/1230	Human	History not known		
<b>DENV2 immune sera</b>	DT001	Human	1996	Sri Lanka	9 years
	IRB019	Human	1997	Thailand	8 years
	DT031	Human	1997	South Pacific	8 years
	DT110	Human	1998	Kuala Lumpur	11 years
	DT134	Human	1984	Yemen	28 years
	BC19	<i>R. macaque</i>	6/13/2011	San Juan, Puerto Rico	12 months
	3F3	<i>R. macaque</i>	6/13/2011	San Juan, Puerto Rico	12 months
	06/94	Human	History not known		
	08/91	Human	History not known		
	08/90	Human	History not known		
<b>DENV3 immune sera</b>	DT105	Human	2002	Thailand	7 years
	DT118	Human	2009	Nicaragua	12 months
	06/267	Human	History not known		
	07/327	Human	History not known		
<b>DENV4 immune sera</b>	DT102	Human	2007	Honduras	2 years
	06/301	Human	History not known		
	07/333	Human	History not known		
	06/105	Human	History not known		
	6F8	<i>R. macaque</i>	6/13/2011	San Juan, Puerto Rico	12 months
	6G0	<i>R. macaque</i>	6/13/2011	San Juan, Puerto Rico	12 months

**Table 2.3. Summary of DENV immune sera used in this study.** Non-human primate (*R. macaque*) immune sera described in previous publication (88).

Sera	ID	DENV2 virion depletion			DENV2 rEDIII depletion		
		BSA-depleted (FRNT <sub>50</sub> )	DENV2-depleted (FRNT <sub>50</sub> )	% loss of neut. (mean ± stdev)	BSA-depleted (FRNT <sub>50</sub> )	rEDIII-depleted (FRNT <sub>50</sub> )	% loss of neut. (mean ± stdev)
DENV2 infection sera	DT001	147	82	44%	171	131	23%
	IRB019	931	261	72%	372	305	18%
	DT031	1288	721	44%	1493	1140	24%
	DT110	644	76	88%	1718	729	58%
	DT134	235	20	91%	168	129	23%
	08/90	192	64	67%	468	357	24%
	average <sup>§</sup>			68% ± 21%			28% ± 15%
DENV2 vaccine sera	250.01.02	148	<20	100%	136	228	0%
	250.01.05	478	37	92%	423	331	22%
	250.01.19	318	<20	100%	272	<20	100%
	average			97% ± 5%			41% ± 53%
All sera	average <sup>§</sup>			78% ± 22%			32% ± 29%

**Table 2.4. Neutralization of rDENV4/2 by human immune sera depleted of DENV2 or EDIII binding antibodies.** Vero-81 cell-based Focus Reduction Neutralization Test (FRNT) was performed sera depleted of DENV2 or rEDIII binding antibodies, and FRNT<sub>50</sub> (sera dilution factor required to neutralize 50% of infection) values were calculated. % loss of neutralization =  $100 - ((\text{DENV2 or rEDIII-depleted FRNT}_{50} / \text{BSA-depleted FRNT}_{50}) \times 100)$ . <sup>§</sup> There was a statistically significant difference between % loss of neutralization between DENV2 and rEDIII depletions by a two-tailed t-test at P<0.05.

## **CHAPTER 3 – Human dengue virus serotype 2 neutralizing antibodies target two distinct quaternary epitopes**

### **3.1 Summary**

Dengue virus (DENV) infection causes dengue fever, dengue hemorrhagic fever and dengue shock syndrome. It is estimated that a third of the world's population is at risk for infection, with an estimated 390 million infections annually. Dengue virus serotype 2 (DENV2) causes severe epidemics, and the leading tetravalent dengue vaccine has lower efficacy against DENV2 compared to the other 3 serotypes. In natural DENV2 infections, strongly neutralizing type-specific antibodies provide protection against subsequent DENV2 infection. While the epitopes of some human DENV2 type-specific antibodies have been mapped, it is not known if these are representative of the polyclonal antibody response. Using structure-guided immunogen design and reverse genetics, we generated a panel of recombinant viruses containing amino acid alterations and epitope transplants between different serotypes. Using this panel of recombinant viruses in binding, competition, and neutralization assays, we have finely mapped the epitopes of three human DENV2 type-specific monoclonal antibodies, finding shared and distinct epitope regions. Additionally, we used these recombinant viruses and polyclonal sera to dissect the epitope-specific responses following primary DENV2 natural infection and monovalent vaccination. Our results demonstrate that antibodies raised following DENV2 infection or vaccination circulate as separate populations that neutralize by occupying domain III and domain I quaternary epitopes. The fraction of neutralizing antibodies directed to different epitopes differs between individuals. The identification of these epitopes could

potentially be harnessed to evaluate epitope-specific antibody responses as correlates of protective immunity, potentially improving vaccine design.

### **3.2 Author Summary**

Dengue viruses (DENV) are flaviviruses transmitted by mosquitos. There are approximately 390 million DENV infections every year, making dengue virus a major global public health concern. While there is a recently licensed DENV vaccine, it has low efficacy against preventing DENV2 infections. Individuals that are naturally infected with DENV2 generate neutralizing antibodies that can be protective against reinfection with DENV2. By studying three of these neutralizing antibodies, we found that they bind to two different locations on the surface of the virus. Additionally we found that most individuals that were naturally infected with DENV2, have antibodies circulating in their blood that target both of these regions. People who were vaccinated against DENV2 also make antibodies targeting both of these sites, suggesting they might also be protected against DENV2 infection. These studies reveal that human antibodies against DENV2 target the same two regions across multiple individuals. Additionally, for a DENV2 vaccine to be protective, it may be important to elicit antibodies directed to these regions as well.

### **3.3 Introduction**

Dengue virus (DENV) is a single stranded positive sense RNA virus that is transmitted by the *Aedes* mosquito (1). There are four distinct DENV serotypes (DENV1-4), and infection results in a range of symptoms, from fever and rash to the more serious dengue hemorrhagic fever and dengue shock syndrome. Over a third of the world's population is at risk for infection, and there are an estimated 390 million infections yearly (1). A primary infection with DENV results in the induction of serotype cross-neutralizing antibodies which can provide temporary serotype cross-protective immunity that is not maintained (64). Over the course of the following

year, these cross-reactive neutralizing antibodies wane, leaving individuals susceptible to infection by the remaining three heterologous serotypes (12). Serotype-specific neutralizing antibodies are maintained in circulation for decades following exposure and may play a critical role in providing subsequent protection against the infecting serotype (64, 89, 90). While antibodies are known to play a key role in protection against DENV reinfection (91), it has also been shown that CD8<sup>+</sup> T-cells (92, 93), CD4<sup>+</sup> T-cells (94), and other mechanisms of cellular immunity are important for protection (95, 96).

The leading DENV vaccines are tetravalent formulations, designed to elicit independent, hopefully protective, neutralizing antibodies against all four serotypes simultaneously (97). Phase 3 efficacy trials in Asia and Latin America showed that the recently licensed tetravalent vaccine, Dengvaxia, had variable efficacy depending on immune status prior to vaccination and the serotype of infection (74, 75). In mixed populations of susceptibles and DENV-immunes, Dengvaxia was 50-80% efficacious against DENV1, DENV3 and DENV4, but only 35-42% against DENV2 (74, 75). Vaccine efficacy was significantly lower in those persons seronegative to DENV compared to individuals who were DENV seropositive at the time of vaccination (74, 75). Moreover, younger vaccinated individuals were hospitalized for DENV more frequently than their unvaccinated counterparts, suggesting that poor immunogenicity in naïve subjects might place individuals at a greater risk of developing severe disease as antibody levels decline over time (4, 63, 98, 99). Indeed, based on long-term follow up data, Dengvaxia is no longer recommended for use in DENV-naïve individuals (98). The Dengvaxia clinical trials have revealed that even individuals with detectable neutralizing antibodies to a particular serotype experienced vaccine break-through infections indicating the mere presence of antibodies that neutralize infection in cell culture assays is not sufficient for protection (69). Therefore, in addition to the level of neutralizing antibodies to each serotype, it is critical to define other properties of human antibodies potentially responsible for durable, protective immunity.

Importantly, while Dengvaxia contains the structural proteins from DENV, the non-structural proteins are from yellow fever virus. It is therefore possible that sufficient T-cell immunity towards DENV epitopes was not achieved (4, 100). DENV vaccines that contain DENV non-structural proteins might generate a more robust T-cell response, and therefore more closely resemble a natural DENV infection, which results in a protective immune response (101).

The DENV envelope glycoprotein (E) ectodomain, which is comprised of three domains (EDI, EDII and EDIII) is the major target of neutralizing antibodies (5). Two E monomers form a dimer in a head-to-tail arrangement, three dimers form a raft, and thirty rafts (180 monomers) cover the entire surface of the virus (76). Our group has previously characterized components of the antibody response to DENV1, DENV2, DENV3 and DENV4 infection by mapping the epitopes of strongly neutralizing human monoclonal antibodies (hMAbs) (21, 27). Importantly, many of these strongly neutralizing hMAbs target quaternary structure epitopes that form as the envelope glycoprotein is assembled on the virus surface (21, 23, 25, 27, 35, 37, 38, 102). In addition, we have demonstrated that we can transplant these quaternary epitopes between DENV serotypes and maintain their biological functions (20, 21, 23, 103). While determining the properties of individual hMAbs is valuable, complex polyclonal antibody response governs protection against subsequent infection. Importantly, the epitope of a single DENV2 serotype-specific hMAb, 2D22, was shown by our group to be targeted by a large fraction of DENV2 neutralizing antibodies in many, but not all individuals after recovery from DENV2 infections, highlighting the potential role of this epitope in protective immunity (21). Despite this, there are additional DENV2 hMAbs that use other epitopes within E, suggesting that there are potentially multiple neutralizing antibody epitopes for each serotype. The goals of this study are to identify novel neutralizing epitopes in DENV2, to develop robust diagnostic reagents for evaluating epitope specific responses with recombinant DENVs (Figure 3.1) and to evaluate the role of



these novel and previously described epitopes as target of polyclonal serum antibodies induced by natural infections and DENV vaccines.

### 3.4 Results

#### *Human monoclonal antibodies target quaternary epitopes on DENV2*

To characterize the epitopes of DENV2 human monoclonal antibodies (hMAbs), we used a panel of three DENV2-specific, strongly neutralizing hMAbs (Table 3.1). The hMAbs were isolated from two donors infected in geographically distinct locations with different DENV2 genotypes (104). The three hMAbs, 3F9, 2D22 and 1L12, bound to whole DENV2 virus (Figure 3.2A). Recently, it has been reported that human antibodies that strongly neutralize DENVs bind to quaternary structure epitopes displayed on E homo-dimers or higher order surface structures required for virion assembly (21, 25, 27, 105). Consonant with previously published results, DENV2 hMAb 2D22 did not bind rE or rEDIII, confirming the quaternary epitope specificity (Figure 3.2B and C). HMAb 1L12 was similar and did not bind to rE or rEDIII (Figure 3.2B and C). In contrast, hMAb 3F9 weakly bound to rE (Figure 3.2B). Because hMAb 3F9 bound well to DENV2 virions and weakly to rE, it is likely that the epitope is dependent on E protein assembly into virions for optimal display. In a blockade of binding assay, 2D22 interfered with 1L12 for binding to DENV2, suggesting that they recognize proximal or overlapping epitopes on the viral envelope (Figure 3.2D). In contrast, 3F9 only partially blocked the binding of 2D22 (Figure 3.2D) indicating the two hMAbs recognize distinct epitopes on the viral envelope.

#### *HMAbs 2D22 and 1L12 bind to proximal but distinct epitopes*

The cryo-EM structure of hMAb 2D22 Fab in complex with DENV2 has been solved (38) and the footprint of the antibody spans EDIII and EDII of two E molecules forming each homo-dimer. Although 2D22 did not bind rEDIII (Figure 3.2C), the antibody binds and neutralizes a DENV4 virus containing the entire EDIII from DENV2 (rDENV4/2-EDIII) (Figure 3.3) (21, 38).

Introducing a single point mutation into this virus (rDENV4/2-EDIII R323G), previously identified as a 2D22 escape mutation (27), (Figure 3.3A), resulted in a loss of binding and neutralization (Figure 3.3B and C), confirming 2D22 uses the transplanted EDIII region. HMAb 1L12, which was isolated from a different donor, showed nearly identical phenotypes, where it gained binding and neutralization to rDENV4/2, indicating that it uses EDIII as part of its complex quaternary epitope (Figure 3.3D and E). Similarly, the R323G mutation in rDENV4/2 results in complete loss of 1L12 binding and neutralization (Figure 3.3D and E).

In addition to binding highly conserved residues in EDII, cryo-EM studies predict that hMAb 2D22 interacts with eight (307, 309, 310, 316, 318, 362, 363, 364) surface-exposed amino acids in DENV2 EDIII (38), five (307, 309, 316, 362, 364) of which differ between DENV2 and DENV4. To refine the map coordinates of 2D22 and 1L12 epitopes, we generated a new EDIII recombinant virus in which these five amino acids in DENV4 were replaced with those from DENV2 (rDENV4/2-EDIII 5aa) (Figure 3.4A). 2D22 was able to partially bind and neutralize this virus at high concentrations of antibody (Figure 3.4B and C). Because the gain in function is only partial, these data suggest that the epitope requires other critical residues in EDIII for maximal binding and neutralization. In contrast, hMAb 1L12 did not bind or neutralize rDENV4 – EDIII 5aa (Figure 3.4D and E), suggesting that its epitope overlaps with 2D22 but engages a different set of residues on EDIII.

### *3F9 targets a complex EDI epitope*

Competition assays with 2D22 indicated that 3F9 binds to an epitope that has minimal if any overlap with 2D22 or IL12 (Figure 3.2D). To map the epitope of hMAb 3F9, we evaluated its binding to a panel of chimeric recombinant DENVs (rDENVs) with alterations in specific domains (Figure 3.5A). Our group has previously shown that strongly-neutralizing hMAbs for DENV1 and DENV3 use the EDI/II hinge region in their epitope (20, 27, 35, 37). hMAb 3F9

bound to a DENV2 virus that had the EDI/II hinge residues replaced with those from DENV4 (rDENV2/4-EDI/II), suggesting it does not use this region in its epitope (Figure 3.5B). Conversely, hMAb 3F9 lost most binding to and neutralization of a DENV2 with 11 of its EDI residues replaced with those from DENV4 (rDENV2/4-EDI), suggesting 3F9 uses an epitope that contains the replaced residues located in EDI (Figure 3.5B and C). To further characterize the 3F9 epitope, we tested its binding to and neutralization of a DENV4 virus that contained 22 surface-exposed EDI residues from DENV2 (rDENV4/2-EDI) (Figure 3.5D). 3F9 bound to and neutralized the EDI transplant virus, confirming EDI as the main target of this hMAb (Figure 3.5E and F), however gain of binding was not complete, suggesting there are other residues required for maximal binding. Our data underscore the importance of cryo-EM analyses to help elucidate the complete 3F9 binding epitope.

In summary, these studies define the location of epitopes recognized by DENV2 type-specific neutralizing hMAbs 2D22, 1L19 and 3F9. Both 2D22 and 1L19 bind to proximal but distinct quaternary epitopes centered on EDIII. hMAb 3F9, on the other hand, binds to an epitope on EDI of E protein.

#### *DENV2 polyclonal neutralizing antibodies target epitopes defined by hMAbs*

To determine if epitopes defined using hMAbs were targets of polyclonal serum neutralizing antibodies, we first performed competition (blockade of binding) assays with human immune sera and hMAbs. Convalescent immune sera from primary DENV2 cases effectively blocked the binding of 2D22 to its epitope (Figure 3.6A). Under identical conditions of treatment, DENV1 or DENV3 immune sera did not block 2D22 from binding, confirming that primary DENV2 infection elicited a 2D22-like serotype-specific antibody response (Figure 3.6A). The same DENV2 immune sera also blocked 3F9 from binding to its epitope, whereas control DENV1 and DENV3 sera did not (Figure 3.6B). Remarkably, the ratio of antibodies targeting the

two epitopes appeared to differ across individuals. Two individuals (DT001 and DT158) were more effective at blocking 2D22 binding than 3F9 binding, whereas DT134 and DT155 were more effective at blocking 3F9 than 2D22 (Figure 3.6A and B), suggesting these individuals had different ratios of antibodies targeting each epitope. DENV2 monovalent vaccine sera also blocked 2D22 and 3F9 binding to their respective epitopes (Figure 3.6C and D), indicating that natural infection and monovalent DENV2 vaccine-elicited antibodies that targeted these epitopes. Interestingly, no DENV2 sera samples were able to completely inhibit either 2D22 or 3F9 from binding to their epitopes, suggesting a limit in the amount these blocking antibodies are present in the sera.

Next, we performed studies to determine if 2D22 and 3F9 epitopes were targets of DENV2 neutralizing serum antibodies. Epitope exchanged recombinant viruses not only provide an approach to map hMAbs, but they can also be used to quantify epitope-specific neutralizing antibodies in immune sera. To measure the amount of neutralizing antibodies targeting 2D22 and 3F9 epitopes, we evaluated the ability of polyclonal DENV2 immune sera (10 samples) or vaccine sera (9 samples) to neutralize rDENV4/2-EDIII (Figure 3.3A) and rDENV4/2-EDI (Figure 3.5D) viruses. Consistent with previous results (21), a large fraction of DENV2 neutralizing antibodies tracked with DENV2 EDIII displayed on the rDENV4/2-EDIII virus (Figure 3.7A). Interestingly, most individuals also had neutralizing antibodies that tracked with the DENV2 EDI epitope displayed on the rDENV4/2-EDI virus (Figure 3.7A). In some individuals (e.g. DT155) there are similar levels of neutralizing antibodies that target both epitopes, whereas in other individuals (e.g. DT128) few if any neutralizing antibodies target the 3F9 EDI epitope (Figure 3.7A). In individuals that received a monovalent DENV2 vaccine, the majority of their neutralizing antibodies target EDIII with a much smaller fraction of the response targeting the EDI epitope (Figure 3.7B). Overall, there is higher tracking of DENV2 specific responses with both the EDIII and EDI epitopes (80% and 54% respectively) in the natural infection sera, as

compared with the vaccine sera (69% and 30% respectively), suggesting vaccination elicits a slightly different antibody response (Table 3.2).

### 3.5 Discussion

People infected with DENVs develop robust and durable antibody responses that contribute to protection against re-infection against the homologous serotype; however, rare instances of re-infection with the same serotype do occur (106). Antibodies that neutralize DENVs in cell-culture assays have been considered to be surrogates of protective immunity *in vivo*. However, this assumption has been challenged by recent results from DENV vaccine trials. Most notably, people who received a tetravalent live attenuated DENV vaccine and developed neutralizing antibodies experienced DENV2 breakthrough infections (63). Breakthrough infections were also documented with the other serotypes despite the presence of neutralizing antibodies (63). This landmark vaccine trial has established that the presence of cell-culture neutralizing antibodies identified using FRNT assays, is not predictive of protection. Indeed, breakthrough DENV infections of vaccinated seronegative children underscore the urgency to understand the essential mechanisms of immune protection in DENV. Moving forward, we need to define key epitopes on DENVs targeted by neutralizing and potentially protective antibodies and develop assays to measure both the level and the molecular specificity of neutralizing antibodies.

In this study, we used a panel of hMAbs, human DENV polyclonal immune sera, and recombinant DENVs (Figure 3.1) to map the location of epitopes recognized by DENV2 neutralizing antibodies. First, we used three DENV2 type-specific and strongly neutralizing hMAbs to map epitopes. hMAbs 2D22 and 1L12 isolated from different people had similar properties and recognized overlapping quaternary epitopes centered on EDIII. Recently Fibriansah *et. al.* determined the cryo-EM structure of 2D22 bound to DENV2 and demonstrated

that the footprint of the 2D22 spanned EDIII and EDII of two E proteins forming a single homodimer (38). Our data indicating that 2D22 recognizes an EDIII centered quaternary epitope are entirely consistent with the footprint determined by Fibriansah *et. al.* We suspect that 1L12 also binds a similar but not identical epitope because of subtle differences in the binding of 2D22 and 1L12 noted in this study. These findings highlight the importance of cryo-EM studies with 1L12, which would provide a more comprehensive view of this larger DENV2 antigenic site. Nevertheless, our observation that two individuals infected with different DENV2 genotypes produced type-specific neutralizing hMAbs targeting a similar region suggests that EDIII is a dominant target of DENV2 neutralizing antibodies. The DENV1, 3 and 4 type-specific, neutralizing hMAbs identified to date do not map to the regions defined by 2D22 and 1L12 indicating that major targets to type-specific neutralizing Abs can differ between serotypes. However, several DENV serotype cross-neutralizing hMAbs that bind across the E homo-dimer have been described recently (25). While these E dimer-dependent epitope (EDE) hMAbs partially overlap with the 2D22 epitope, they recognize patches that are highly conserved between serotypes unlike 2D22.

HMAb 3F9 and 1L12, which were isolated from the same person, have distinct epitopes, consistent with bivalent recognition of the EDIII and EDI DENV epitopes in most DENV polyclonal immune sera. The 3F9 epitopes is centered on EDI at a site that overlaps with known DENV1 and DENV4 neutralizing hMAbs (35, 80, 107). Therefore, unlike 2D22, the region recognized by 3F9 is targeted by type-specific neutralizing antibodies to other serotypes as well.

Our previous work demonstrated that a majority of the polyclonal antibody response following DENV2 infection and vaccination appeared to be directed to a quaternary EDIII epitope (21). In some individuals however, neutralization titers did not track as strongly with this epitope, suggesting that two or more neutralizing epitopes are targeted disproportionately after

primary DENV2 infections. We propose that the EDI epitope defined by the hMAb 3F9 represents a second major neutralizing epitope on DENV2. Most individuals with naturally acquired DENV2 infections contained antibodies targeting both epitopes however some individuals targeted only one epitope, or had a skewed response. Similar results were observed in DENV2 vaccinated individuals, where there were antibodies targeting each epitope, however the overall response is dominant to the EDIII epitope. Overall, there was a higher response of antibodies tracking with the EDIII than the EDI epitope in both the natural infection and vaccinated sera (Table 3.2). Interestingly, some individuals had complete neutralizing antibody responses tracking with both epitopes, suggesting that they potentially generated redundant populations of antibodies. Generating populations of antibodies directed to different regions on E could be an important component of an effective antibody response. Viruses can mutate to escape antibody pressure, but simultaneously escaping antibody pressure to multiple sites on E would be more challenging (108, 109). As some individuals appear to mount preferential responses to one site or the other after natural infection or vaccination, it is possible that strains with natural variation within one of these epitopes may allow for repeat or breakthrough DENV2 infections.

Without a clear understanding of what constitutes a protective DENV antibody response to each serotype, it is challenging to evaluate current DENV vaccines. By defining the epitopes targeted by DENV2 hMAbs and polyclonal sera, we hope to determine if there are antibody based correlates of protection and use these to evaluate current vaccines in the pipeline, and inform the design of next-generation vaccines. Using recombinant DENVs that contain both gain of function and loss of function epitopes, we can rapidly map in high-throughput assays the epitopes of large panels of hMAbs, prioritizing targets for crystallographic studies and downstream analyses.

### **3.6 Methods**

#### *Virus Construction*

Recombinant viruses were constructed using a four-cDNA cloning strategy. The DENV genome was divided into four fragments, and subcloned into separate cDNA plasmids with unique type IIS restriction endonuclease cleavage sites at the 5' and 3' ends of each fragment. A T7 promoter was introduced into the 5' end of the A fragment. Plasmid DNA was grown in *Escherichia coli* cells, digested with the corresponding enzymes, gel purified, ligated together with T4 DNA ligase and transcribed with T7 polymerase to generate infectious genome-length capped viral RNA transcripts. RNA was electroporated into C6/36 cells, cell culture supernatant containing virus was harvested and passaged onto C6/36 cells to generate a passage one virus stock.

#### *Cells*

C6/36 cells (ATCC CRL-1660) were grown in Gibco minimal essential medium (MEM) at 32°C. Vero-81 cells (ATCC CCL-81) were maintained in Dulbecco's modified Eagle's medium (DMEM) at 37°C. Media were supplemented with fetal bovine serum (FBS) (10% for Vero-81 and 5% for C6/36) which was lowered to 2% after infection. C6/36 media were supplemented with nonessential amino acids. All media were additionally supplemented with 100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml Amphotericin B. All cells were incubated in 5% CO<sub>2</sub>.

#### *Ethics Statement*

Human dengue immune sera used in this study were obtained from a previously described Dengue Traveler collection at University of North Carolina, and were all primary DENV2 natural infections (21, 23, 27). Vaccine sera were obtained from individuals who received a live-attenuated monovalent DENV2 vaccine as developed by the US National



Institutes of Health (NIH) and were provided by Anna Durbin and Stephen Whitehead. All human sera samples were obtained under Institutional Review Board approval and were anonymized.

#### *Virus Titration and Immunostaining*

One day prior to inoculation, 24-well cell culture plates were seeded with either  $5 \times 10^4$  Vero-81 cells. Virus stocks were serially diluted 10-fold then added to cells (after growth media was removed) for one hour at 37°C. After incubation, cells were overlaid with 1% methylcellulose in OptiMEM I (Gibco) supplemented with 2% FBS, nonessential amino acids and 100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml Amphotericin B, and incubated at 32°C. After four days incubation, overlay was removed, cells were washed with phosphate-buffered saline (PBS) and fixed in 80% methanol. Cells were blocked in 5% non-fat dried milk (blocking buffer) then incubated with anti-prM MAb 2H2 and anti-E MAbs 4G2 diluted in blocking buffer. Cells were washed with PBS, then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Sigma) diluted in blocking buffer. Plates were washed and foci were developed using TrueBlue HRP substrate (KPL).

#### *Binding Enzyme-Linked Immunosorbent Assay (ELISA)*

For whole DENV ELISA, plates were coated with 100ng/well mouse MAb 4G2 and 2H2 overnight at 4°C. Plates were washed with Tris-buffered saline with 0.05% Tween (TBST) and blocked in 3% non-fat dried milk in TBST (blocking buffer), and equal quantities of virus (as previously titrated by ELISA using cross-reactive polyclonal DENV immune sera) were added and incubated for 1 hour. For rE and rEIII ELISA, plates were directly coated with protein and incubated. Plates were washed and primary human MAbs were diluted in blocking buffer and added to plate for 1 hour. Plates were washed and alkaline phosphate (AP)-conjugated secondary antibodies were added for 1 hour. Plates were washed, developed using p-

nitrophenyl phosphate substrate and color changes were quantified by spectrophotometry. Assays were developed until OD values were within linear range of the assay, therefore absolute OD values may vary between graphs. All binding assays are based on two experiments performed in duplicate.

#### *Blockade of Binding Assay*

Plates were coated with antibody, blocked, and virus was captured as described above. DENV polyclonal immune sera were depleted of cross-reactive antibodies as described previously (23). Briefly, sera were incubated with beads coated with purified DENV4 antigen, then beads were pelleted to removed cross-reactive antibodies bound to bead:antigen complexes. Cross-reactive depleted sera were then diluted 1:10 in blocking buffer and incubated for 1 hour. Plates were washed and alkaline phosphate (AP)-conjugated 2D22 (100ng/well) or 3F9 (50ng/well) were added for 1 hour. Plates were developed as described above. Percent blockade was calculated as follows =  $(100 - [\text{OD of sample} / \text{OD of negative control}] * 100)$ . Blockade of binding assays are based on two experiments performed in duplicate.

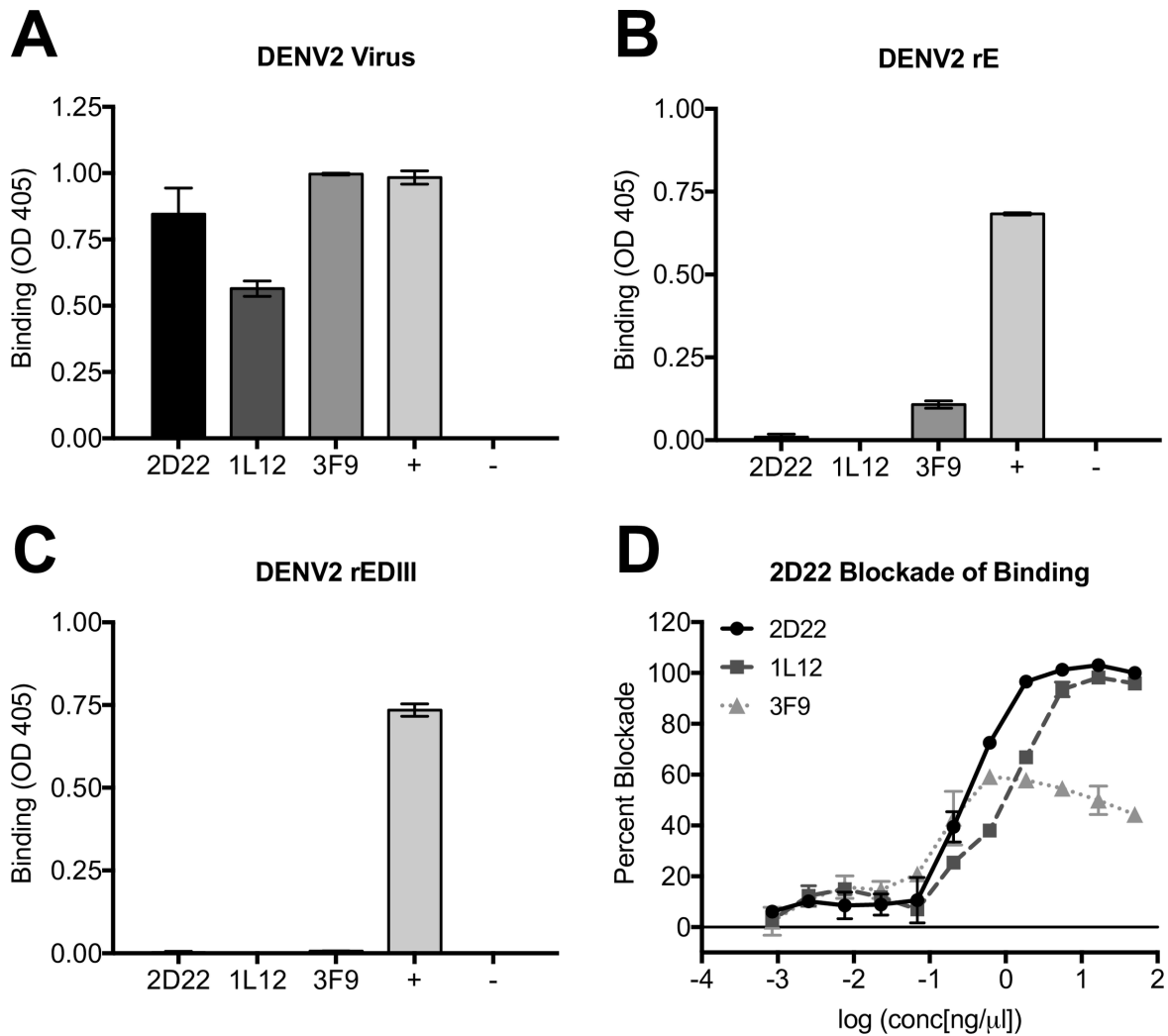
#### *Focus Reduction Neutralization Test*

For the focus reduction neutralization test (FRNT), hMAbs were diluted 4-fold and mixed with ~45 focus-forming units (FFU) of virus, and incubated for 1 hour at 37°C. After incubation, virus:hMAb mixture was added to Vero-81 cells for 1 hour at 37°C or C6/36 cells for 1 hour at 32°C, then overlay was added and cells were incubated and fixed and stained as described above. Foci were counted and FRNT<sub>50</sub> titers were calculated as the concentration of antibody or sera dilution factor required to neutralize 50% of the virus. Neutralization assays are based on two (HMAbs) or one (immune sera) experiments performed in triplicate.

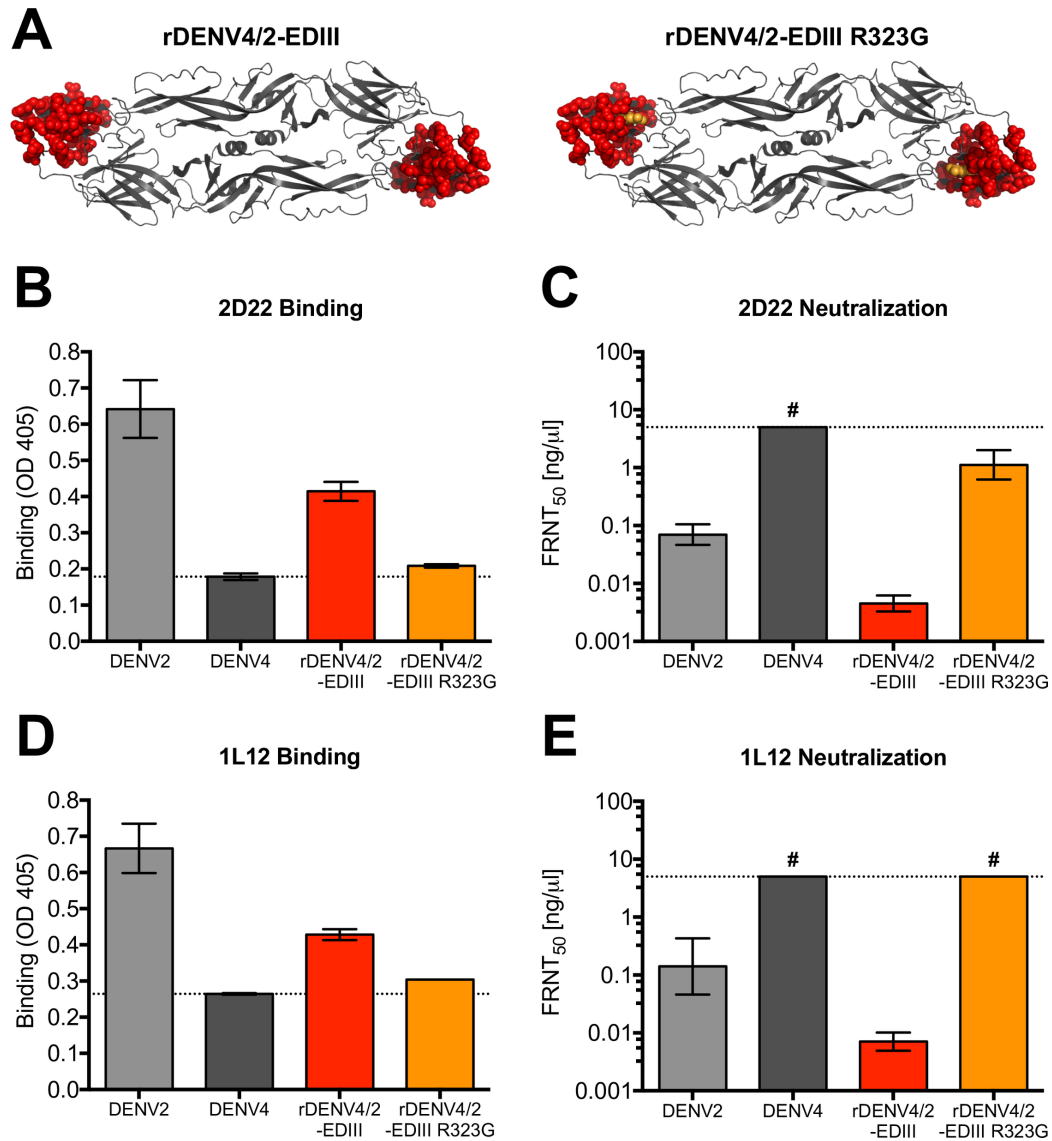
		Residue																																							
		ED/II																																							
Parental Virus	Virus Name	46	49	52	53	55	58	124	126	128	129	139	147	148	156	157	160	161	162	163	167	169	170	171	173	174	176	177	180	199	205	210	222	270	271	272	273	277	278	293	294
DENV4	DENV4	T	T	E	V	L	T	K	T	N	L	V	D	T	S	N	V	T	A	T	R	P	S	V	V	K	P	D	E	M	T	K	A	I	Q	M	S	L	L	R	I
	rDENV4/2-EDIII	T	T	E	V	L	T	K	T	N	L	V	D	T	S	N	V	T	A	T	R	P	S	V	V	K	P	D	E	M	T	K	A	I	Q	M	S	L	L	R	I
	rDENV4/2-EDIII R323G	T	T	E	V	L	T	K	T	N	L	V	D	T	S	N	V	T	A	T	R	P	S	V	V	K	P	D	E	M	T	K	A	I	Q	M	S	L	L	R	I
	rDENV4/2-EDIII 5aa	T	T	E	V	L	T	K	T	N	L	V	D	T	S	N	V	T	A	T	R	P	S	V	V	K	P	D	E	M	T	K	A	I	Q	M	S	L	L	R	I
DENV2	rDENV4/2-EDI	I	E	E	V	L	T	K	T	N	L	I	E	E	G	K	K	E	I	K	Q	S	I	T	A	E	T	G	T	M	T	K	A	I	Q	M	S	L	L	Q	L
	DENV2	I	E	Q	P	T	K	N	E	K	V	I	E	E	G	K	K	E	I	K	Q	S	I	T	A	E	T	G	T	L	A	R	P	V	D	S	G	H	M	Q	L
	rDENV2/4-EDI/II	I	E	E	V	L	T	K	T	N	L	I	E	E	G	K	K	E	I	K	Q	S	I	T	A	E	T	G	T	M	T	K	A	I	Q	M	S	L	L	Q	L
	rDENV2/4-EDI	I	E	Q	P	T	K	N	E	K	V	I	E	E	G	K	V	T	A	T	Q	S	S	V	V	K	P	D	E	L	A	R	P	V	D	S	G	H	M	Q	L

		Residue																																							
		EDIII																																							
Parental Virus	Virus Name	300	303	307	309	312	320	322	323	325	329	331	335	337	340	342	343	345	346	348	351	353	354	355	357	358	359	360	361	362	364	365	369	379	383	384	385	386	388	390	393
DENV4	DENV4	T	S	S	D	M	T	V	K	K	A	A	V	I	R	V	N	E	K	V	V	S	S	T	L	A	E	N	T	N	V	T	L	V	G	N	S	A	T	H	R
	rDENV4/2-EDIII	S	T	K	V	I	I	I	R	Q	D	S	I	F	T	L	E	R	H	L	L	T	V	N	I	V	T	E	K	D	P	V	A	I	E	P	G	Q	K	N	K
	rDENV4/2-EDIII R323G	S	T	K	V	I	I	I	G	Q	D	S	I	F	T	L	E	R	H	L	L	T	V	N	I	V	T	E	K	D	P	V	A	I	E	P	G	Q	K	N	K
	rDENV4/2-EDIII 5aa	T	S	K	V	M	T	V	K	Q	A	A	V	I	R	V	N	E	K	V	V	S	S	T	L	A	E	N	T	D	P	T	L	V	G	N	S	A	T	H	R
	rDENV4/2-EDI	T	S	S	D	M	T	V	K	K	A	A	V	I	R	V	N	E	K	V	V	S	S	T	L	A	E	N	T	N	V	T	L	V	G	N	S	A	T	H	R
DENV2	DENV2	S	T	K	V	I	I	I	R	Q	D	S	I	F	T	L	E	R	H	L	L	T	V	N	I	V	T	E	K	D	P	V	A	I	E	P	G	Q	K	N	K
	rDENV2/4-EDI/II	S	T	K	V	I	I	I	R	Q	D	S	I	F	T	L	E	R	H	L	L	T	V	N	I	V	T	E	K	D	P	V	A	I	E	P	G	Q	K	N	K
	rDENV2/4-EDI	S	T	K	V	I	I	I	R	Q	D	S	I	F	T	L	E	R	H	L	L	T	V	N	I	V	T	E	K	D	P	V	A	I	E	P	G	Q	K	N	K

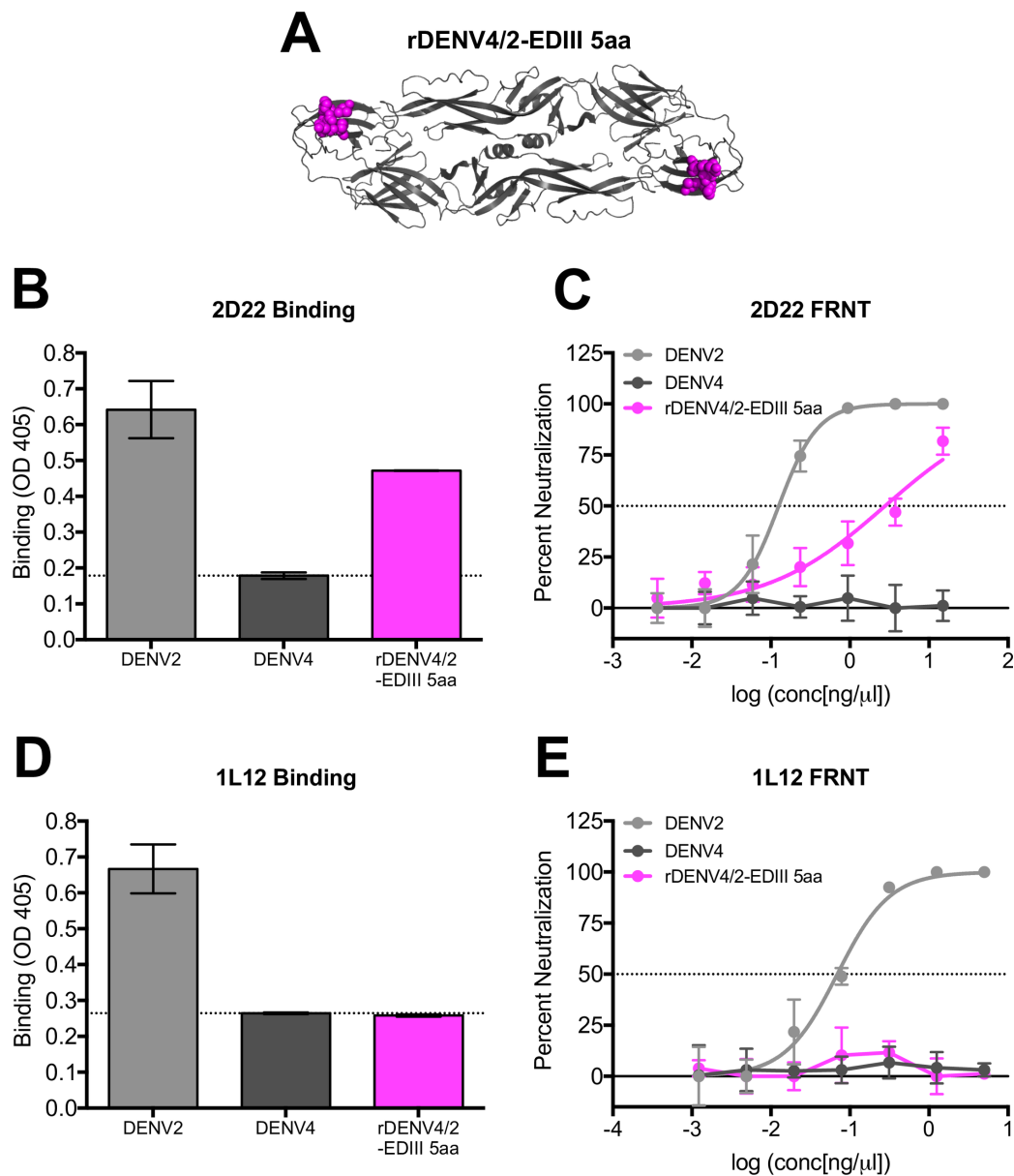
**Figure 3.1. Sequences of rDENVs.** Amino acid sequences of WT and rDENVs used to map antibody epitopes.



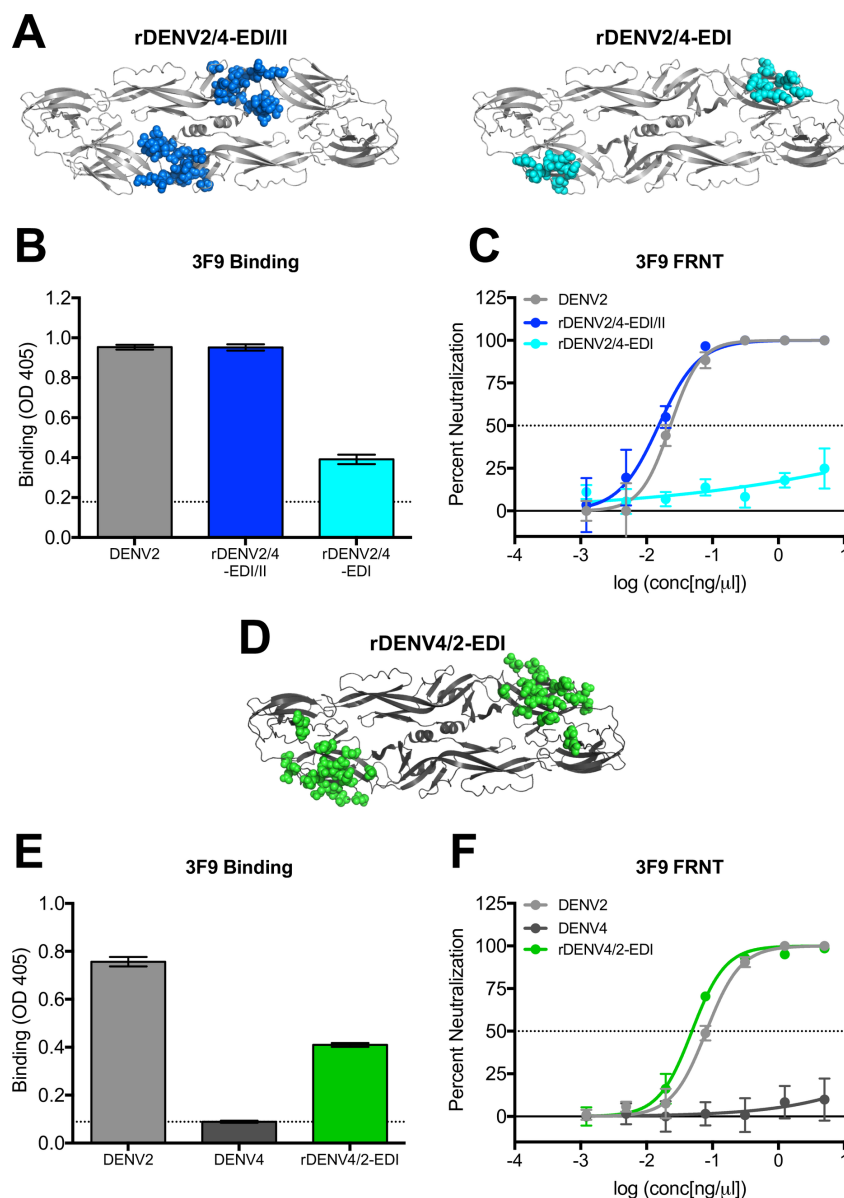
**Figure 3.2. DENV2 serotype-specific hMAbs use multiple quaternary epitopes.** DENV2 hMAbs 2D22, 1L12 and 3F9 were assessed for their ability to bind whole DENV2 virions (A), DENV2 rE (B), and DENV2 rEDIII (C). Positive control (+) hMAb is DVC10.16, a DENV2 hMAb that uses a simple A-strand epitope contained entirely within EDIII and 5J7 is a DENV3 serotype-specific hMAb as a negative control (-). (D) Blockade of binding assay where hMAbs 2D22, 1L12 or 3F9 were assessed for their ability to block 2D22-AP from binding to DENV2.



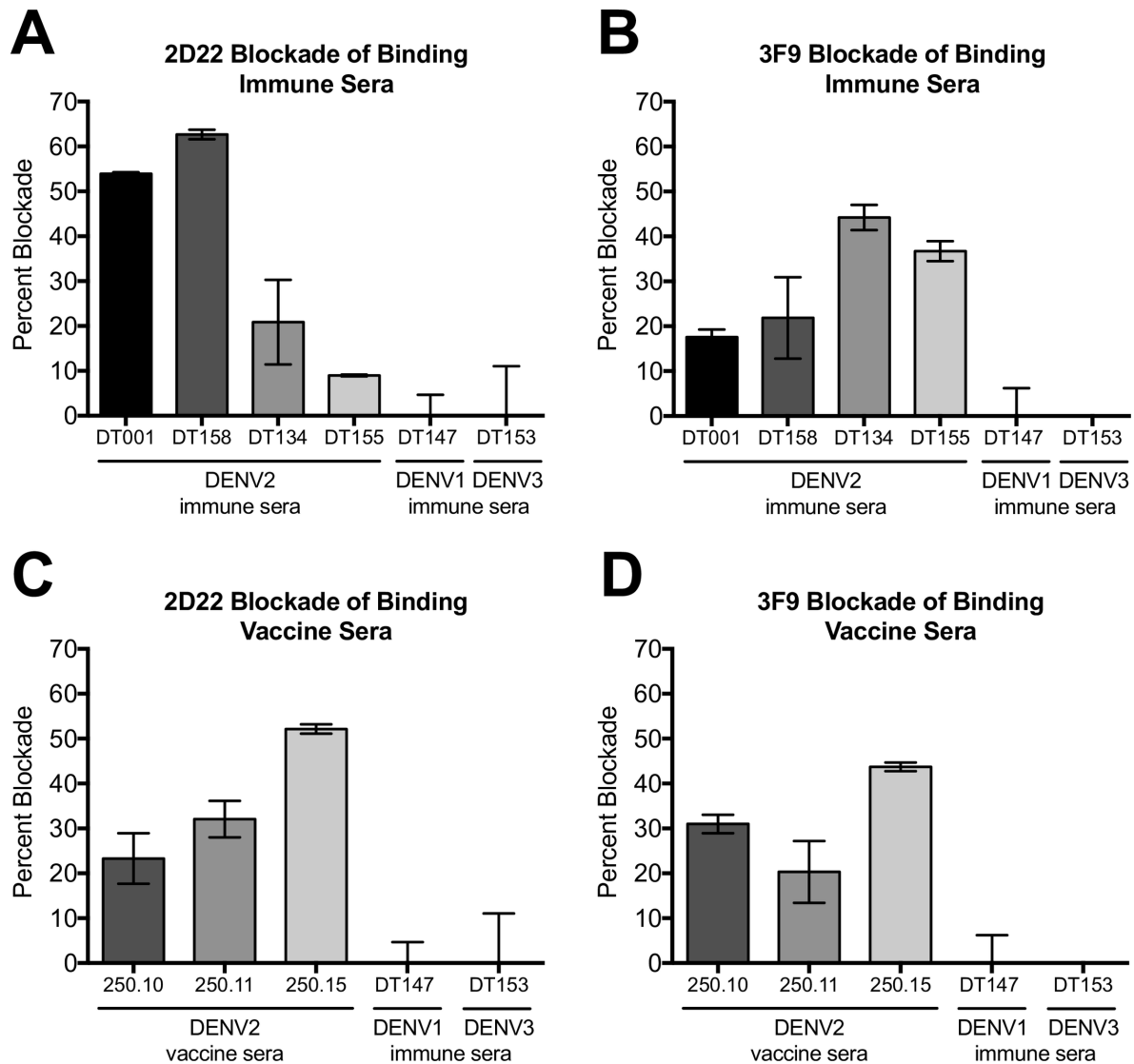
**Figure 3.3. HMABs 2D22 and 1L12 use EDIII in their epitopes.** (A) rDENV4/2-EDIII is DENV4 virus containing entire EDIII from DENV2. rDENV4/2-EDIII R323G is rDENV4/2 virus with single point mutation at residue 323. 2D22 and 1L12 were assessed for their ability to bind (B and D) and neutralize (C and E) recombinant DENVs in ELISA binding assays and Vero-81 Focus Reduction Neutralization Tests (FRNT). Dotted line in ELISA represents the background signal. FRNT<sub>50</sub> represents the concentration of antibody required to neutralize 50% of infection. # = virus was not neutralized at highest concentration of hMAb tested (5ng/μl).



**Figure 3.4. HMAbs 2D22 and 1L12 use different critical residues in their epitopes.** (A) rDENV4/2-EDIII 5aa is a DENV4 virus with five EDIII residues from DENV2. 2D22 and 1L12 were assessed for their ability to bind (B and D) and neutralize (C and E) recombinant DENV in ELISA binding assays and Vero-81 Focus Reduction Neutralization Tests (FRNT). Dotted line in ELISA represents the background signal, determined as the OD value of wells containing all reagents except for viral antigen.

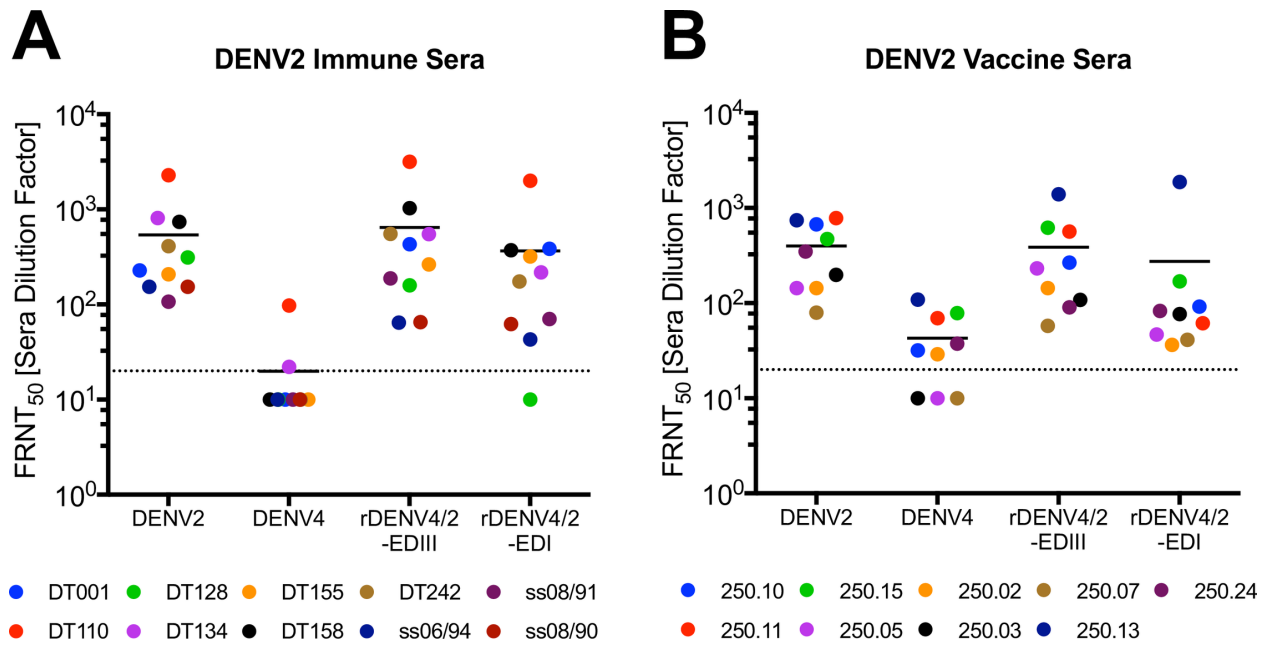


**Figure 3.5. HMAb 3F9 use an epitope contained within EDI.** (A) rDENV2/4-EDI/II is DENV2 virus containing EDI/II hinge region residues from DENV4. rDENV2/4-EDI is DENV2 virus containing EDI residues from DENV4. 3F9 was assessed for its ability to bind (B and E) and neutralize (C and F) recombinant DENVs in ELISA binding assays and Focus Reduction Neutralization Tests (FRNT) in Vero-81 cells (C) or C6/36 cells (F). Dotted line in ELISA represents the background signal. (D) rDENV4/2-EDI is DENV4 virus containing EDI residues from DENV2.



**Figure 3.6. DENV2 polyclonal antibodies target EDIII and EDI epitopes.** Blockade of binding assay where DENV2 natural infection immune sera (A, B) or DENV2 monovalent vaccine sera (C, D), were assessed for their ability to block 2D22-AP (A, C) or 3F9-AP (B, D) from binding to their respective epitopes on DENV2. DENV immune sera were depleted of cross-reactive antibodies prior to blockade assay.





**Figure 3.7. DENV2 polyclonal neutralizing antibodies target two distinct epitopes.** DENV2 immune sera (A) and monovalent vaccine sera (B) were evaluated for their ability to neutralize WT and rDENV in C6/36 Focus Reduction Neutralization Tests (FRNT). Dotted line represents limit of detection (20), samples with no neutralization were plotted at one half the limit of detection (10). Y-axis indicates sera dilution factor required to neutralize 50% of virus.

Donor	Infection Location	Infecting Genotype	Infection Year	MAb	Neut <sub>50</sub> (µg/ml)			
					DENV 1	DENV 2	DENV 3	DENV 4
IRB019	Thailand	Asian	1997	2D22	>10	0.08	>10	>10
DT001	Sri Lanka	Cosmopolitan	1996	1L12	>10	0.4	>10	>10
				3F9	>10	0.03	>10	>10

**Table 3.1. DENV2 monoclonal antibodies.** Virus was isolated from subject DT001, sequenced and found to be part of the cosmopolitan genotype (110). Subject IRB019 was infected in Thailand in 1997 when the DENV2 Asian genotype strain was circulating in the region.

Type	Sera ID	FRNT <sub>50</sub> [Sera Dilution Factor] <sup>α</sup>				Percent tracking with <sup>β</sup>	
		DENV2	DENV4	rDENV4/2-EDIII	rDENV4/2-EDI	EDIII	EDI
Natural Infection Sera	DT001	228	---	431	385	100	100
	DT110	2286	97	3170	2004	100	88
	DT128	313	---	158	---	51	0
	DT134	809	22	550	217	65	24
	DT155	208	---	263	320	100	100
	DT158	740	---	1030	372	100	50
	DT242	410	---	553	175	100	43
	ss06/94	153	---	65	43	42	28
	ss08/91	107	---	188	70	100	66
	ss08/90	154	---	65	62	42	41
	Average (±SD)					80% ± 27	54% ± 34
DENV2 Monovalent Vaccine Sera	250.10	675	32	267	92	35	9
	250.11	783	69	566	62	63	0
	250.15	473	79	621	169	100	19
	250.05	144	---	232	47	100	33
	250.02	144	29	144	36	80	5
	250.03	199	---	108	77	55	39
	250.07	80	---	58	41	72	52
	250.13	743	109	1396	1871	100	100
	250.24	348	38	90	83	15	13
	Average (±SD)					69% ± 30	30% ± 31

**Table 3.2. Percent polyclonal neutralization tracking with each epitope.** <sup>α</sup> FRNT<sub>50</sub> [Sera Dilution Factor] were calculated as the sera dilution factor required to neutralize 50% of the virus, extrapolated from Figure 3.7. <sup>β</sup> Percentage calculated as = (rDENV4/2-EDIII FRNT<sub>50</sub> – DENV4 FRNT<sub>50</sub>)/(DENV2 FRNT<sub>50</sub>) x 100, and = (rDENV4/2-EDI FRNT<sub>50</sub> – DENV4 FRNT<sub>50</sub>)/(DENV2 FRNT<sub>50</sub>) x 100.

## **CHAPTER 4 – Human DENV2-specific antibody response following tetravalent dengue vaccination**

### **4.1 Introduction**

#### *Dengue vaccines*

An effective dengue virus (DENV) vaccine should result in an immune response that is protective against infection to each of the four DENV serotypes. The leading DENV vaccines are live attenuated, tetravalent formulations designed to elicit an independent, robust antibody response to each serotype simultaneously. However, replication interference between the different vaccine components, and immune dominance has led to an unbalanced or skewed response (111). Individuals with an unbalanced response might may be poorly protected against some serotypes. These individuals might be at increased risk for severe disease following a natural DENV infection, through mechanisms of antibody dependent enhancement (ADE).

Sanofi Pasteur's live-attenuated, tetravalent DENV vaccine, Dengvaxia, is based on the yellow fever 17D vaccine. The chimeric vaccine, CYD-TDV (chimeric yellow fever dengue-tetravalent dengue vaccine) contains the prM and E structural components of each DENV serotype, in an attenuated yellow fever virus vaccine backbone (YFV17D) (68). The vaccine contains equal amounts of each serotype component, and is given in three doses, six months apart. Preclinical testing of CYD-TDV *in vitro*, and *in vivo* in both mice and non-human primate models, showed that the vaccine was both safe and immunogenic (112-114). Early phase I and II clinical trials evaluated safety and immunogenicity in flavivirus-naïve or pre-immune healthy

individuals (toddlers to adults) before the vaccine was moved into larger phase IIb and III trials (115-119).

#### *Antibody response to Dengvaxia vaccination*

Early studies evaluated the neutralizing antibody responses to vaccination using cell culture based assays. The vaccine stimulated broad neutralizing responses against all four serotypes (115-118), however the properties of these antibodies, such as their serotype-specificity, was not known. Additionally, flavivirus pre-immune individuals had higher, and broader neutralizing responses (119). More recent work has looked at evaluating the molecular specificity of antibodies induced by the Dengvaxia vaccine in healthy trial participants (22). Flavivirus-naïve healthy adults vaccinated with three doses of Dengvaxia, generated a robust cross-neutralizing response against all four serotypes, with highest neutralization titers to DENV4, followed by DENV2, DENV3 and DENV1 (22). These results were consonant with Sanofi's clinical testing of their vaccine neutralizing antibody responses, and promising that the vaccine would have high efficacy in phase III trials.

Cell-culture based neutralization assays however, cannot distinguish between neutralization due to serotype-specific neutralizing antibodies, and cross-reactive antibodies. Additionally, it is thought that weakly neutralizing cross-reactive antibodies can overestimate neutralization capacity via cell culture assays, whereas they are known to not be protective in people. Using depletion-based assays, the fraction of the neutralization response attributable to serotype-specific antibodies and cross-reactive antibodies can be calculated, by specifically removing certain populations of antibodies from the serum. Briefly, beads are coated with purified DENV, incubated with polyclonal immune sera, and then centrifuged to pellet bead-DENV-Ab complexes, selectively removing any antibodies that bind to the virus (70). For example, to determine the contribution of DENV1 serotype-specific antibodies to total

neutralization of DENV1, beads are coated with a mix of DENV2, DENV3 and DENV4 antigen, which will bind serotype-specific antibodies targeting DENV2, DENV3 and DENV4, as well as all cross-reactive antibodies, leaving only DENV1 serotype-specific antibodies in the serum sample. We can then measure neutralization of DENV1 with undepleted and depleted sera, and calculate the percent of serotype-specific antibodies contributing to neutralization.

When these experiments and analyses were performed with Dengvaxia vaccine sera, it was discovered that DENV4 neutralization was primarily due to serotype-specific antibodies, whereas DENV1, DENV2 and DENV3 were primarily targeted by cross-reactive antibodies (22). Vaccination of individuals who were flavivirus pre-immune, resulted in higher neutralization titers against all four serotypes, predominantly due to boosting of cross-reactive antibodies (22). Additionally, pre-immune individuals had higher post-vaccination neutralization titers compared with flavivirus naïve individuals (22). These analyses indicate that in naïve individuals, the vaccine induces a robust DENV4 serotype-specific antibody response that should be protective in people. The highly cross-reactive response against serotypes DENV1, DENV2, and DENV3 might not be sufficient from protection following natural exposures. Importantly, in flavivirus pre-immune individuals, the vaccine appears to boost a cross-reactive response, similarly to a secondary DENV infection, which should be protective against natural infection.

#### *Results from Dengvaxia phase II/III trials*

A proof-of-concept phase IIb clinical efficacy trial (CYD23b) was performed in schoolchildren aged 4-11 in Thailand to evaluate vaccine efficacy (120). It was found that overall vaccine efficacy was ~30%, but differed greatly by serotype, with the worst protection against DENV2 (3.5% vaccine efficacy), which dominated during the trial (2.6% total attack rate, 43% of breakthrough infections were DENV2) (120). Importantly, cell culture neutralization titers following the third dose, were high and similar across all four serotypes (highest titers against

DENV2), revealing that the mere presence of cell-culture based neutralizing antibodies is not predictive of, nor sufficient for protection from natural infection (120). Despite the low overall vaccine efficacy, and essential failure against DENV2, the vaccine was determined to be well-tolerated, with no evidence of safety signals one year following the third dose. This study did however, highlight the discordance between cell-culture based neutralization titers and protection, and the highly variable vaccine efficacy based on DENV serotype.

Following the moderate success of the phase IIb trial, large-scale phase III trials were performed in Asia in children aged 2-14 (CYD14) and in Latin America in individuals aged 9-16 (CYD15). Both trials consisted of an active phase for one year following the third vaccine dose, followed by an additional four years of hospital phase. Overall vaccine efficacy was moderate against all four serotypes, ~55% and ~60% for CYD14 and CYD15 respectively (68). Importantly, similar to results from the CYD23b trial, vaccine efficacy varied highly by serotype. Vaccine efficacy reached ~80% efficacy against DENV4 in CYD15, but efficacy against DENV2 was always the lowest in both CYD14 (35%) and CYD15 (42%) (4, 68). The observed attack rates were slightly higher than CYD23b (4.7% and 2.9% for CYD14 and CYD15 respectively) but unlike CYD23b, serotype distribution of breakthrough infections was evenly balanced between the four serotypes. Therefore, the poor efficacy against DENV2 seen in CYD14 and CYD15 was not driven by a disproportionate amount of circulating DENV2 during these trials; only 23% (CYD14) and 21% (CYD15) of breakthrough infections were DENV2 (68).

Analyses of sera from CYD14 and CYD15 vaccinated individuals revealed that the vaccine elicited neutralizing antibodies to all four serotypes as determined by plaque reduction neutralization assays (4, 63, 68, 69). Importantly, higher neutralizing antibody titers from month 13 (one month following the third vaccine dose) correlated with a higher vaccine efficacy and significantly lower risk of virologically confirmed DENV (VCD) infection (69). Individuals with

high levels of neutralizing antibodies however, still experienced breakthrough infections for all four serotypes, suggesting that the presence of neutralizing antibodies are not sufficient for protection (69). Additionally, higher neutralizing antibody titers strongly correlate with baseline serostatus, potentially driving the higher vaccine efficacy seen in individuals who were pre-immune to DENV (68). In all trials, vaccine efficacy was lowest in individuals who were DENV-naïve prior to vaccination. These results raise the important distinction between the CYD-TDV boosting pre-existing immunity in baseline seropositive individuals, leading to high vaccine efficacy in this population, versus the ability of CYD-TDV to elicit a protective immune response in naïve individuals (4, 68).

In DENV endemic countries, age is a strong correlate for previous DENV infection. Results from CYD14 and CYD15 analyses revealed that the vaccine performed worse in both younger and naïve populations (68). Additionally, it was found that in CYD14, there was a 7.45 relative risk for hospitalization for VCD in younger children, suggesting that Dengvaxia sensitizes seronegatives to more severe disease in subsequent natural infection (63, 121). These results revealed that in this population, receiving the vaccine put individuals at a significantly higher risk for more severe disease upon natural infection compared to non-vaccinated controls (121). These results are supported by the hypothesis that the vaccine elicits weakly neutralizing cross-reactive antibodies (22), and upon natural infection, these antibodies enhance infection and disease via antibody dependent enhancement (ADE) mechanisms (4, 121, 122). While these results fully support the hypothesis that, baseline immune status, and not merely young age, is a crucial factor for vaccine success or risk, Dengvaxia was licensed for use in individuals over the age of 9, regardless of serostatus (123). Dengvaxia was licensed in 19 countries, the Philippines government bought 3 million doses of Dengvaxia, and intended to vaccinate one million 9-year old school children. By December of 2017, over 800,000 children had received at least one dose of Dengvaxia (122).



Unfortunately, with additional years of follow-up study of CYD14 and CYD15, it was revealed that in certain populations, namely DENV-naïve individuals, Dengvaxia resulted in increased risk of more severe disease and hospitalization following natural infection (98, 99, 124). Importantly, these results were in agreement with short-term follow up results (63, 68), revealing that Dengvaxia sensitizes seronegatives to enhanced disease, regardless of vaccination age. These results prompted the WHO and Sanofi to change their recommendations for the vaccine to only be used in individuals over 9 years of age who are baseline seropositive to DENV (98, 99, 124). In the Philippines there are multiple reports of deaths of Dengvaxia-vaccinated children after subsequent natural DENV infection; the Philippines Department of Health has formed a committee to investigate whether the deaths were linked to Dengvaxia vaccination (122).

#### *Outstanding Research Questions*

Early results from Dengvaxia's phase III trials suggested that an effective DENV vaccine was possible, however the longer-term follow-up results have greatly complicated this story. Importantly, these trial results reveal that our current cell-culture based antibody neutralization titer correlates of protection, are insufficient to estimate protective immunity (66, 67). Additionally these results reveal that currently used neutralization assays, do not distinguish between strongly and weakly neutralizing serotype-specific antibodies, which are known to be important for protection, and weakly cross-neutralizing antibodies, which are known to lead to ADE in people (70, 89, 125). Evaluating the quantity and quality of serotype-specific antibody responses to each of the four serotypes following vaccination, could improve, or provide more accurate antibody based metrics of protection. While these analyses are critical for evaluating responses to all four serotypes, vaccine efficacy in all trials was dramatically worst against DENV2, so the subsequent approach will focus on that serotype.

## 4.2 Approach and Results

### *Determine quality of Dengvaxia induced DENV2 neutralizing antibodies*

It is known that natural DENV infection results in a population of strongly neutralizing serotype-specific antibodies, which play a critical role in protection. Previous work revealed that vaccination of healthy, flavivirus-naïve individuals, resulted in high DENV2 neutralization titers, but that neutralization was predominantly due to cross-reactive antibodies (22). It is possible that there is a certain percentage or titer of DENV2 serotype-specific antibodies required to protect against infection. To test this hypothesis, we are performing depletion assays using the following samples; 1) DENV2 natural infection sera, 2) CYD14/CYD15 month 13 sera from vaccinated individuals. Within the CYD14/15 collection, we will evaluate i) samples with DENV2 breakthrough infections, or ii) samples from matched vaccinated controls without breakthrough infections. While we will not know if individuals without DENV2 breakthrough infections were exposed and protected, or simply unexposed, we can evaluate any qualitative trends between the groups. By performing depletion assays, we can determine if there are any trends between percentage or absolute titer of DENV2 serotype-specific and breakthrough infection or not. We predict that, consistent with analyses from earlier trials (22), the vaccine will elicit low levels of DENV2 serotype-specific antibodies in both breakthrough and non-breakthrough individuals, in comparison to the high levels we see with DENV2 natural infection.

Pilot studies using depletion and neutralization assays with sera from vaccinated naïve healthy adults allow us to calculate DENV2 serotype-specific neutralization titers, and percent of DENV2 neutralization from serotype-specific antibodies. Vaccine sera were either control depleted with BSA, or depleted of cross-reactive antibodies using DENV4, and neutralization assays are performed against DENV2 and DENV4 viruses. Using a primary DENV2 infection sera as a positive control, we can calculate the relative loss of neutralization due to cross-reactive antibodies, and find that up to 60% of the total neutralization of DENV2 comes from

serotype specific antibodies (Figure 4.1). Using these same assays with vaccine sera from naïve individuals, we find a range of absolute DENV2 neutralization titers (FRNT<sub>50</sub> of 149-398) and percent of DENV2 serotype-specific antibodies (18-62%) (Figure 4.2).

We were curious to see if there was a potential correlation between absolute DENV2 neutralization (by serotype-specific and cross-reactive antibodies) and percent serotype-specific neutralization. We see that there is no correlation however, and that despite absolute titers being similar, there is a large range of percent serotype-specific neutralization (Figure 4.3). Interestingly, the sample with the lowest titer (CYD17-52) had the highest percentage of serotype-specific antibodies (62%), which is similar to the percentage seen in a DENV2 natural infection (Figure 4.1).

#### *Determine epitopes targeted by DENV2 serotype-specific antibodies*

Serotype-specific antibodies can be further characterized based on the types of epitopes they target. Polyclonal immune sera can contain many antibodies targeting simple or linear epitopes. Depleting sera of these antibodies has minimal impact on total neutralization, revealing that these antibodies contribute little towards the robust neutralization capacity of polyclonal immune sera (27, 28, 65). Conversely, antibodies targeting quaternary epitopes, spanning either envelope domains, monomers or dimers, and present only on the intact virion, provide the strong neutralization by serotype-specific antibodies in polyclonal immune sera (27). We have identified and characterized two distinct, quaternary DENV2 epitopes (21, 126). One of these epitopes, defined by the human DENV2 monoclonal antibody (MAb) 2D22, spans across EDIII or one monomer into EDII fusion loop region of the neighboring monomer (38). The other epitope, defined by MAb 3F9 uses a quaternary epitope on EDI (126). The epitopes defined by these antibodies, are targeted by strongly neutralizing DENV2 serotype-specific polyclonal

antibodies from individuals naturally infected with DENV2, and are therefore, thought to important epitopes to target for protection (126).

Importantly, these epitopes are transplantable between serotypes, and have both been moved into a DENV4 virus (126). These viral reagents allow us to measure the amount of DENV2 serotype-specific antibodies targeting these epitopes in polyclonal immune sera. Using these viruses, and the three types of sera samples described above (natural infection and vaccinees with DENV2 breakthrough infection or without) we can measure the amount and percentage of DENV2 serotype-specific antibodies targeting each of these two epitopes. It is already known the individuals with DENV2 natural infections target both epitopes, albeit the ratio targeting each varies across individuals (126). These analyses will allow us to determine if Dengvaxia elicits antibodies targeting either or both epitopes, and whether there are any trends between epitope and breakthrough or protection.

Using the same samples, we were able to perform neutralization assays using a rDENV4 virus containing EDIII from DENV2, which we've previously shown to track with the human monoclonal antibodies 2D22, 1L12, and polyclonal DENV2 neutralizing antibodies (21, 126). We find that all of the DENV2 serotype-specific neutralization tracks with this epitope (Figure 4.4), despite the large range of percent serotype-specific antibodies. This result is consistent with DENV2 natural infection sera (21, 126), suggesting that while the vaccine might elicit lower levels of serotype-specific antibodies, they appear to target the same region.

#### *Impact of antigenic diversity on vaccine response*

Within each DENV serotype, there is genetic and antigenic diversity resulting in multiple distinct genotypes (104, 127). In natural DENV infection, it is thought that the serotype-specific antibody response is broad enough, that it would protect against reinfection with any genotype

within the homologous DENV serotype (104, 127). There is growing evidence however, that antigenic diversity within serotypes might be great enough to escape pre-existing immunity (128). Additionally, reinfection with homologous serotype is rare, but does occasionally occur, especially after clade replacements altering the circulating genotype (106). An effective DENV vaccine would not only need to elicit strongly neutralizing antibodies to each serotype, but the neutralizing response would need to have enough breadth to neutralize all circulating DENV genotypes, not just the genotype of the vaccine strains.

It was proposed that the poor efficacy of Dengvaxia could be partially due to circulating genotypes that differ from the vaccine strains (4). Early evaluation of CYD-TDV responses tested the ability of vaccinated non-human primate sera to neutralize a large panel of DENVs, representing many of the genotypes within each serotype (129). Importantly, they found that vaccine sera neutralizes the majority of DENV stains tested, however they saw the most variability within DENV2, with only half of strains efficiently neutralized within 95% confidence interval of the assay (129). This result suggested that there is broad genotypic breadth within the antibody response to vaccination, but importantly, these assays do not distinguish between neutralization by serotype-specific antibodies and cross-reactive antibodies. Therefore, it is possible the breadth of response they see (to all serotypes and genotypes), might be driven by a highly cross-reactive antibody response.

In Dengvaxia phase III trials, viruses from all breakthrough infections (from both vaccinees and placebos) were isolated and sequenced to determine the genetic epidemiology of these trials (130). For DENV2, the circulating genotypes were Asian I and Cosmopolitan (CYD14), and Asian/American (CYD15). The circulating Cosmopolitan genotype had the most amino acid diversity (97.4% conservation) from the vaccine strain (Asian I), whereas the circulating Asian/American and Asian I strains were more similar (97.9% conservation for both)

(130). Considering these subtle differences in circulating strain sequences, the vaccine had no observable significant differences in vaccine efficacy against different genotypes (130). This is in great contrast to DENV4, where the vaccine works significantly better against vaccine-matched circulating strains (DENV4 genotype II) versus against vaccine mismatched strains (DENV4 genotype I) (130). These results are in line with analyses that determined the quality of antibody responses following vaccination; cross-reactive neutralizing antibodies drive DENV1-3 neutralization, whereas DENV4 neutralization is primarily due to serotype-specific neutralizing antibodies (22). Antibodies that recognize multiple serotypes are less likely to be impacted by amino acid diversity within each serotype, whereas serotype-specific antibodies would be more sensitive to genotypic variation.

Given that experiments determining breadth of vaccine sera neutralization were performed with sera containing both cross-reactive and serotype-specific antibodies, it is possible that DENV2 serotype-specific antibodies are more sensitive to genotypic variation. To test this hypothesis, we will use DENV2 natural infection sera (as a control) and CYD14/15 vaccine sera from both individuals with and without DENV2 breakthrough infections. We will deplete sera of cross-reactive antibodies, so only DENV2 serotype-specific neutralizing antibodies are remaining, and test ability of depleted sera to neutralize a large panel of DENV2 genotypic variants. We suspect that undepleted sera will be highly cross-neutralizing to all strains, unlike DENV2-specific sera, which might show genotypic-specific variation, likely with the highest neutralization titers against Asian I vaccine-matched strains.

As a proof-of-concept, we have performed similar experiments testing the importance of DENV4 genotypic variation (Figure 4.5). The DENV4 serotype is comprised of five genotypes; I, IIa, IIb, III, IV (sylvatic), and V (Figure 4.5A). We have generated an isogenic panel of DENV4 viruses containing the envelope glycoprotein of each genotype and used them in neutralization

assays. We find that there is a large range of neutralization against a panel of primary DENV4 natural infection sera (Figure 4.5B). These results reveal that even following natural infection, while there is large breadth of neutralization against all genotypes, some viruses are consistently neutralized either better or worse. By applying these same techniques to DENV2 and Dengvaxia vaccine sera, we will be able to measure any genotype-specific differences in the antibody response to vaccination.

### **4.3 Conclusions**

Early analyses of CYD-TDV suggested it should be successful in large clinical trials because in human trials it elicited antibodies that were strongly neutralizing to all four serotypes (68). As our understanding of the complex adaptive immune response to DENV infection evolves, the metrics used to evaluate vaccine responses has evolved as well. At one point, merely the presence of DENV neutralizing antibodies, as determined through binding or cell culture based assays (a common correlate for many other vaccines, such as hepatitis B, rotavirus and tetanus), was thought to be sufficient as a correlate of protection against DENV. We now know the quality of neutralizing antibodies, either serotype-specific or cross-reactive, and perhaps epitope specificity of the response, is important in protective immunity. Additionally, the epitopes targeted by strongly neutralizing antibodies can be a determinant for their ability to be neutralizing or enhancing following natural infection. Given the evolving metric of antibody-based correlates of protection for DENV, new experiments and analyses of Dengvaxia vaccine sera are anticipated to predict the success and failure of the vaccine in different populations. Importantly, these new experiments and analyses can be applied to other vaccine platforms as well (both Takeda and NIH currently have DENV vaccines in phase III clinical trials) to potentially better predict vaccine efficacy (67). Additionally, results of Dengvaxia's phase III clinical trials have reveal the importance of basic research to fully define the adaptive immune

response to natural DENV infection, before fully defining metrics of protection, which are critical in evaluating vaccine responses.

#### **4.4 Methods**

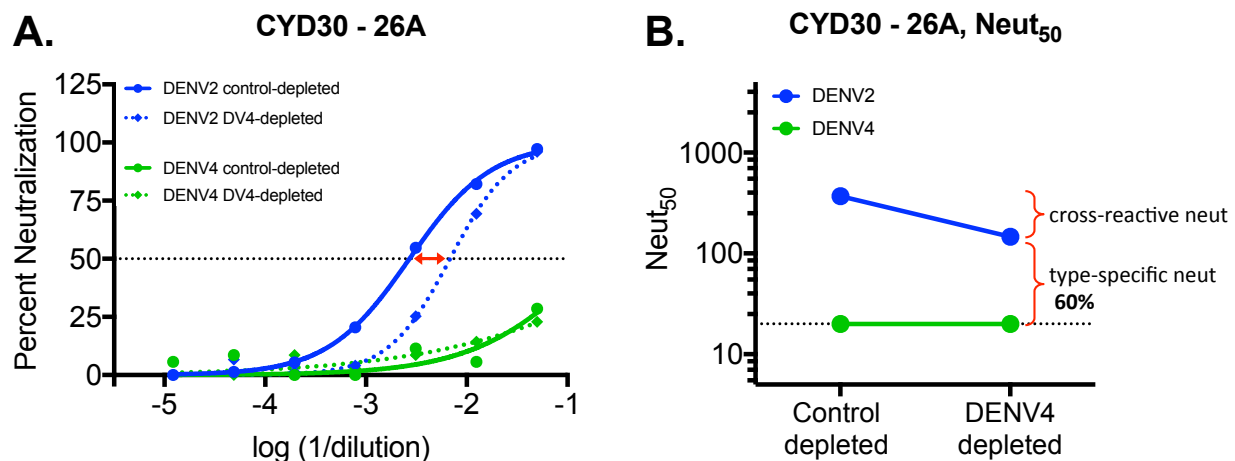
##### *Depletion assays to remove cross-reactive antibodies*

Polyclonal immune sera were depleted of cross-reactive antibodies as previously described (27). Briefly, polystyrene beads were coated with either BSA or purified DENV4 antigen. Polyclonal immune sera were incubated with bead:antigen complexes at 37°C for 45 minutes, and repeated two additional times with new bead:antigen mix. Confirmation of complete depletion of antibodies was performed using an ELISA.

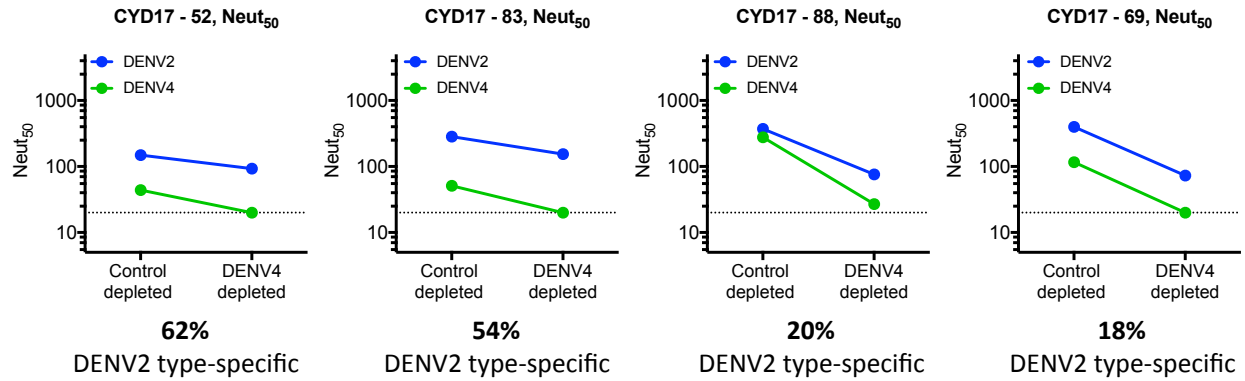
##### *Focus reduction neutralization assay (FRNT)*

Neutralization assays were performed as previously described (21, 126). Briefly, polyclonal immune sera were diluted 4-fold and mixed with virus, Ab:virus complex was incubated at 37°C for one hour, then added to confluent Vero cells. After one hour, overlay was added and cells were incubated for 4-5 days, then fixed and immunostained as previously described (21, 126).

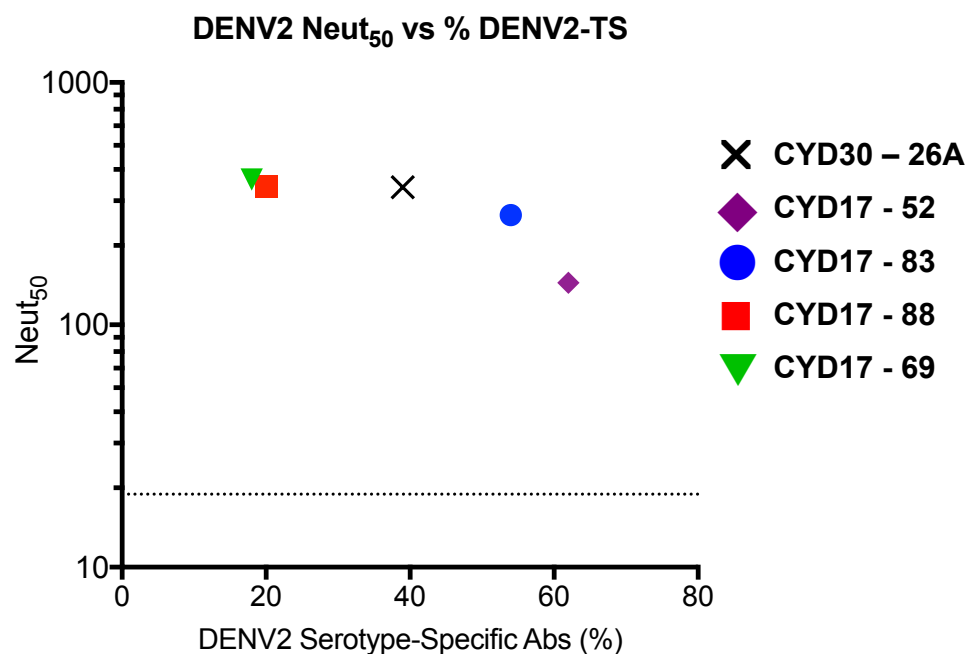




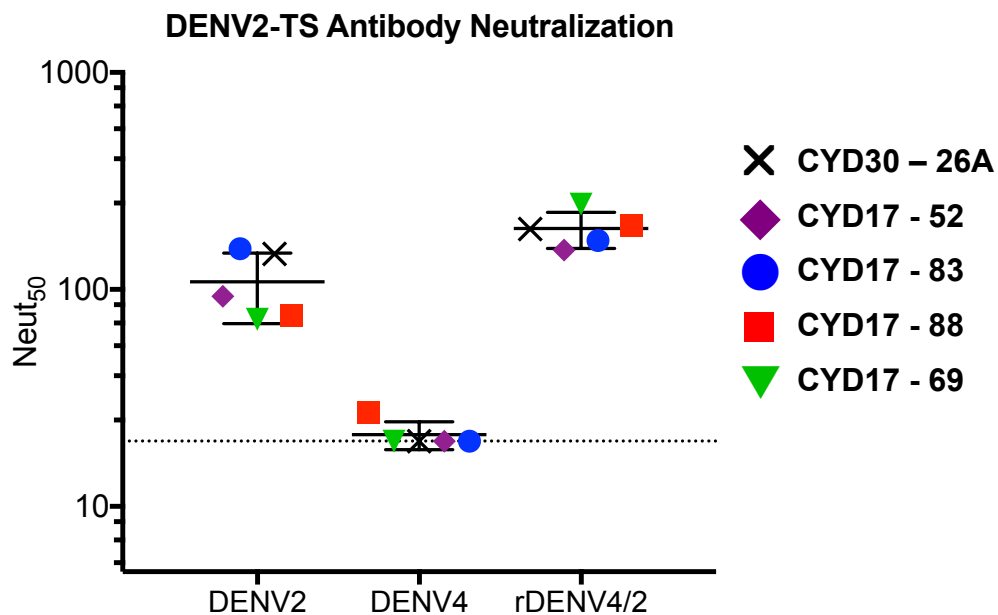
**Figure 4.1. DENV2 polyclonal immune sera contains high levels of serotype-specific antibodies.** A) Focus reduction neutralization test was performed using DENV2 (blue) and DENV4 (green) viruses with either BSA-depleted (solid line) or DV4-depleted (dashed line) sera from a DENV2 immune individual. B) Neut<sub>50</sub> (serum dilution factor required to neutralization 50% of virus) values are extrapolated from full neutralization curves, and percent of neutralization from serotype-specific antibodies is measured.



**Figure 4.2 Dengvaxia immune sera contains varying amount of DENV2 serotype-specific antibodies.** Dengvaxia immune sera were either control depleted or depleted of cross-reactive antibodies (DENV4-depleted) and depleted sera was using in neutralization assays against DENV2 and DENV4, and the Neut<sub>50</sub> (serum dilution factor required to neutralization 50% of virus) values were calculated. Percent of serotype-specific neutralization was calculated from Neut<sub>50</sub> values.

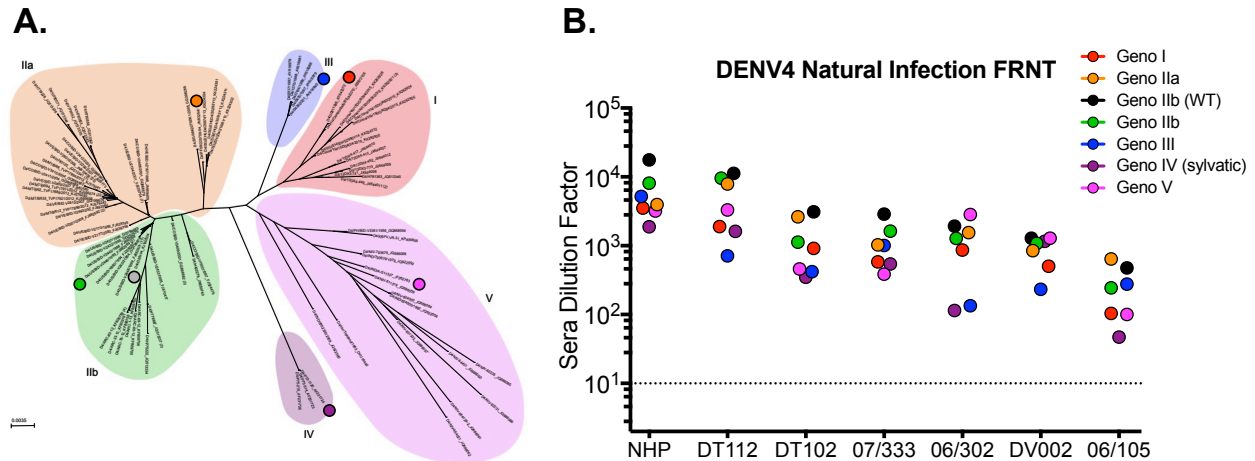


**Figure 4.3 DENV2 neutralization titer does not correlate with percentage of serotype-specific antibodies.** DENV2 neutralization titers (serum dilution factor required to neutralization 50% of virus) were plotted against the percentage of neutralization due to serotype-specific antibodies.



**Figure 4.4 Dengvaxia-elicited DENV2 serotype-specific antibodies target an EDIII epitope.**

DENV2 serotype-specific sera (depleted of cross-reactive antibodies) were evaluated for their ability to neutralize DENV2, DENV4, and rDENV4/2, a chimeric DENV4 virus that contains EDIII from DENV2.  $Neut_{50}$  values are calculated as the serum dilution factor required to neutralization 50% of virus.



**Figure 4.5 DENV genotypic variation can impact polyclonal neutralization.** A) The DENV4 serotype is composed of five genetically distinct genotypes, with genotype II further divided into Ila and Iib. A phylogenetic tree was generated using envelope protein amino acid sequences using the neighbor-joining method. B) rDENVs containing the envelope protein of each genotype were tested for their ability to be neutralized by a panel of DENV4 primary immune sera. Neut<sub>50</sub> values are calculated as the serum dilution factor required to neutralization 50% of virus.

## **CHAPTER 5 – Epitope addition and ablation via manipulation of a DENV1 infectious clone**

### **5.1 Summary**

Despite the clinical relevance, dengue virus (DENV) research has been hampered by the absence of robust reverse genetic systems to manipulate the viral serotypes for propagation and generation of mutant viruses. In this manuscript, we describe application of an infectious clone system for DENV serotype 1 (rDENV1ic). Similar to previous clones in both flaviviruses and coronaviruses, the approach constructs a panel of contiguous cDNAs that span the DENV genome and can be systematically and directionally assembled to produce viable, full-length viruses. Comparison of the infectious clone derived virus with the original isolate reveals identical sequence, comparable end-point titers, and similar foci staining. Both focus forming assays and percent infection by flow cytometry revealed overlapping replication levels in two different cell types. Moreover, serotype-specific monoclonal antibodies (MAbs) bound similarly to infectious clone and the natural isolate. Using the clone, we were able to insert a DENV4 type-specific epitope recognized by primate MAb 5H2 into envelope (E) protein domain I (EDI) of DENV1 and recover a viable chimeric recombinant virus. The recombinant DENV1 virus was recognized and neutralized by the DENV4 type-specific 5H2 MAb. The introduction of the 5H2 epitope ablated two epitopes on DENV1 EDI recognized by human MAbs (1F4 and 14C10) that strongly neutralize DENV1. Together, the work demonstrates the utility of the infectious clone

and provides a resource to rapidly manipulate the DENV1 serotype for generation of recombinant and mutant viruses.

## **5.2 Importance**

Dengue viruses (DENVs) are significant mosquito transmitted pathogens that cause widespread infection and can lead to severe infection and complications. Here we further characterize a novel and robust DENV serotype 1 (DENV1) infectious clone system that can be used to support basic and applied research. We demonstrate how the system can be used to probe the antigenic relationships between strains by creating viable recombinant viruses that display or lack major antibody epitopes. The DENV1 clone system and recombinant viruses can be used to analyze existing vaccine immune responses and inform second-generation bivalent vaccine designs.

## **5.3 Introduction**

Dengue viruses (DENV) represent a significant threat to human health and the global economy causing widespread and frequent epidemics (1, 131). Transmitted by female mosquitos, the DENV family has four known serotypes that are genetically and antigenically related. Infection with any one DENV serotype typically produces asymptomatic or mild disease, but induces long-term immunity against that serotype (132). However, subsequent exposure to a different serotype may result in more severe disease characterized by dengue hemorrhagic fever or dengue shock syndrome often attributed to antibody dependent enhancement (2, 131). Overall, potential adverse disease outcomes due to immune cross-reactivity complicates efforts to produce effective vaccines against the DENV family (133).

To study aspects of infection and pathogenesis, a number of reverse genetic systems have been generated for members of the flavivirus family, which includes DENV (134, 135).

These approaches have provided powerful tools to manipulate the viral genome and have given insight into viral protein function, virulence determinants, viral immune evasion, host specificity, and other virus/host interactions (134). Reverse genetic systems have also facilitated improved research methodologies with the development of reporter assays as well as means to produce virus like particles and live attenuated vaccines (136, 137). In addition, infectious clones provide the opportunity to explore a clonal population of DENV with relatively few mutations versus the “mutant spectrum” typically observed in laboratory passaged stocks (138-140). Together, these tools have been critical in building a foundation to understanding DENV disease and pathogenesis.

Yet, while current systems have been useful, several obstacles remain for DENV reverse genetic systems (134). Flavivirus clones are difficult to maintain in bacteria and often result in sequence rearrangements or mutations that reduce expression of toxic elements from the viral genome. Several approaches have been employed to improve propagation including use of low copy plasmid (134), insertion into bacterial or yeast artificial chromosomes (141-143), disruption of promoter regions in viral cDNA (144), as well as microbial free propagation approaches (145, 146). However, these methods often diminish yields, produce changes in viral sequence, or require significant extraneous effort that reduces the overall utility of the approaches. Importantly, many of these reverse genetic systems report significant attenuation relative to their control wild-type viruses despite similar consensus sequencing (134). Overall, these results highlight the obstacles for using reverse genetic systems to study DENV disease and pathogenesis.

In this study, we extend our characterization of a dengue virus serotype 1 (DENV1) infectious clone (20) by comparing and contrasting the growth and antigenic properties of molecularly cloned recombinant and wild-type dengue virus serotype 1 (DENV1). Importantly,



DENV1 derived from the clone maintained equivalent replication and complete antibody binding fidelity with the wild-type strain. Using the tetrapartite DENV1 molecular clone, we then inserted a known DENV4 envelope protein domain I (EDI) neutralization epitope and demonstrated efficient gain of neutralization function. However, by introducing these changes, we simultaneously ablated two known DENV1 antibody binding sites and disrupted their binding and neutralization. Together, the results illustrate the utility of this newly developed infectious clone as a platform to study DENV immunity by exchanging functional complex epitopes between DENV strains, simultaneously gaining new neutralization properties to other strains while ablating the primary neutralizing antigenic sites of DENV1.

## **5.4 Results**

### *Design and construction of dengue 1 full-length infectious clone*

The DENV1 infectious clone (rDENV1ic) was synthesized as a panel of four continuous cDNA segments that span the entire genome (Figure 5.1A) (20). Based on sequence from a laboratory strain of DENV1 West Pac '74, each segment is flanked by class IIS restriction endonuclease sites that cleave palindromic sequences into asymmetric 3 nucleotide overhangs. These overhangs permit directional assembly of the cleaved fragments to generate full-length viral cDNA. With the exception of an ablated restriction site (see methods), the sequence directly reconstitutes the wild-type laboratory strain. The ligated cDNA fragments are then used as a template to produce full-length viral RNA, which is electroporated into C6/36 mosquito cells. To demonstrate transfection efficiencies of full-length transcripts, infectious center assay indicate low, but stable infection (0.4%) (Figure 5.1B). After several days, cell culture supernatant containing progeny rDENV1ic virions is harvested and passaged once on C6/36 cells to generate working virus stocks.

RNA isolated from infected cells was verified relative to the parental wild-type strain using genome-length analysis finding no differences in the consensus amino acid sequences. Similarly, end-point titers from C6/36 cells (Figure 5.1C) found no notable difference between the wild-type and rDENV1ic derived viruses in regards to replication. In C6/36 cells, both wild-type and infectious clone derived virus maintains similar sized foci following infection (Figure 5.1D). Together, the results indicate that no replication deficit nor foci formation deficit was observed between the wild-type and infectious clone.

#### *Replication kinetics of rDENV1ic*

Some of the previously described reverse genetic systems for DENVs have been hampered by replication attenuation relative to the wild-type strain despite identical consensus sequences (134). To determine if rDENV1ic is attenuated relative to the wild-type virus, growth curves were completed in C6/36 mosquito cells over a seven day time course (Figure 5.1E). The data indicates that wild-type and rDENV1ic replicate equivalently, peaking in focus forming units 5 days after infection (Figure 5.1E). Percent infection of C6/36 cells also demonstrated no significant difference between the wild-type and infectious clone with nearly 100% infection observed 4 days after infection (Figure 5.1F). We next examined replication in the human monocyte derived U937 cell line stably expressing DC-SIGN, a known DENV attachment factor (Figure 5.1G); similar to the C6/36 mosquito cell line, we observed no difference in the replication of the wild-type and rDENV1ic in U937+DC-SIGN cells. These results demonstrate no detectable attenuation of the rDENV1ic clone relative to the wild-type strain in either insect or human cells.

#### *The display of antibody epitopes on rDENV1ic*

Antibody binding and neutralization are a critical issue in DENV pathogenesis in humans; as such, the infectious clone and parental WT strain must display the same surface

architecture and epitopes. To compare the display of antibody epitopes, ELISAs were performed using a panel of DENV1-specific monoclonal antibodies against rDENV1ic and the parental wild-type strain (Table 5.1). Monoclonal antibody (MAb) 1F4, derived from a DENV1 infected patient (27), binds in the envelope domain I (EDI) region and extends into the EDI and domain II (EDII) hinge region (84); ELISA with MAb 1F4 produced strong and equivalent binding to both the wild-type and infectious clone (Figure 5.2A). Similarly, the EDI, EDIII and EDI/II hinge region is the epitope for MAb 14C10, which also bound similarly between the wild-type and rDENV1ic (Figure 5.2B). Finally, MAb 1C19.2, an EDIII antibody, also bound similarly to both wild-type and rDENV1ic (Figure 5.2C). Together, the results indicate that rDENV1ic displays the same antibody epitopes as the wild-type control virus.

We also tested the binding of DENV serotype 2, 3 and 4 monoclonal antibodies (Table 5.1) to rDENV1ic. Beginning with a DENV2-specific antibody, MAb 2D22 (38), failed to bind to either wild-type or rDENV1ic (Figure 5.2D). Similarly, MAb 5J7 (37) also fails to bind to rDENV1ic or wild-type strain while efficiently binding DENV3 (Figure 5.2E). Finally, MAb 5H2, an EDI antibody specific to DENV4, showed no binding to either DENV1 iteration (Figure 5.2F). Together, combined with DENV1 monoclonal data, the results indicate that rDENV1ic has the same surface architecture and epitopes as the wild-type strain.

#### *Antibody neutralization of rDENV1ic*

While observing equivalent antibody binding between rDENV1ic and wild-type virus by ELISA, neutralization is an even more sensitive functional assay to compare viruses. Small changes in binding conformation may induce enhancement versus neutralization; as such, these assays must be equivalent between the infectious clone and wild-type to justify the use of rDENVic for vaccine and immunogenicity studies. Using a focus reduction neutralization test (FRNT), we examined the ability of well-defined MAbs to block virus infectivity. For all three

DENV1 monoclonal antibodies (1F4, 14C10, and 1C19.2), percent neutralization was nearly identical between the wild-type and rDENV1ic (Figure 5.3A-C). Similar to ELISA results, monoclonal antibodies against the other DENV serotypes were unable to neutralize either the clone or wild-type virus (Figure 5.3D-F). Extending studies to a U937+DC-SIGN flow-based neutralization assay, percent infection results revealed overlapping neutralization of rDENV1ic and wild-type following DENV1 monoclonal antibody incubations (Figure 5.4A-C). In addition, none of the other DENV serotype specific antibodies provides any significant reduction in viral infection (Figure 5.4D-F). Together, the results match the ELISA data and indicate antibody neutralization fidelity between the infectious clone and wild-type viruses in two independent neutralization assays.

#### *Gain of DENV4 monoclonal binding and neutralization*

To use the rDENV1ic to study antibody responses to specific epitopes, we generated a DENV1 mutant virus that incorporates the DENV4 5H2 epitope into the DENV1 backbone virus. 5H2 is a DENV4 type-specific and strongly neutralizing MAb isolated from a non-human primate infected with DENV4 (80, 147). A high-resolution structure of 5H2 bound to DENV4 E protein has led to the identification of 17 residues within EDI that interact with the antibody (Figure 5.5) (80, 147). Of these 17 amino acids, five are conserved between DENV1 and DENV4. The remaining 12 residues in DENV1 were replaced with those from DENV4, by manipulating plasmid A of the DENV1 infectious clone (rDENV1ic-5H2-epitope, herein referred to as rDENV1ic-EDI) (Figure 5.6A) (Figure 5.5). The DENV4 MAb 5H2 bound and neutralized the rDENV1ic-EDI virus (Figure 5.6B and C). As the parental clone and wild-type DENV1 were not recognized by 5H2 (Figure 5.6B and C), these results confirm successful transfer of a conformational epitope from DENV4 into DENV1.

### *Ablation of DENV1 monoclonal binding and neutralization*

The epitope of DENV4 MAb 5H2 overlaps with the known DENV1 type-specific epitopes recognized by human neutralizing MAbs 1F4 and 14C10 (84). Importantly, 1F4 has over one third of its predicted contact residues replaced in the rDENV1ic-EDI virus (Figure 5.7A and 5.5). Insertion of the DENV4 5H2 epitope partially disrupted the DENV1 1F4 epitope resulting in a 80-fold reduction in  $EC_{50}$ , but not complete ablation in binding of 1F4 to rDENV1ic-EDI (Figure 5.7B). Similarly, neutralization assays indicate that 1F4 nearly lost all of its ability to neutralize the rDENV1ic-EDI mutant (Figure 5.7C). MAb 14C10 has a smaller percentage (15%) of its epitope disrupted in the rDENV1ic-EDI virus (Figure 5.7D and 5.5), as it has additional contact residues in EDIII from the neighboring dimer, that are maintained (Figure 5.8) (36). Despite this smaller percentage, binding of 14C10 is still reduced (Figure 5.7E) and 14C10 neutralization of rDENV1ic-EDI is mostly lost (Figure 5.7F). In contrast, DENV1 monoclonal antibody E103, which targets an EDIII epitope (Figure 5.7G) (148), had no significant reduction in ELISA binding relative to wild-type or the infectious clone (Figure 5.7H). Additionally, MAb E103 maintained robust neutralization indicating maintenance of other DENV1 epitopes within the mutant virus (Figure 5.7I). In summary the introduction of the 5H2 epitope residues to create the rDENV1ic-EDI virus leads to efficient display of a heterologous DENV4 epitope and the disruption of two native DENV1 type-specific epitopes. Our results illustrate the utility of the infectious clone for transferring DENV epitopes between serotypes, and identifies functional consequences on other conserved epitopes within the transferred amino acid regions.

## **5.6 Discussion**

While a number of reverse genetic systems for dengue viruses have been developed, significant obstacles including replication attenuation, sequence alteration, as well as labor and cost intensive techniques have limited their utility (134). In this manuscript, we further characterize a novel DENV1 molecular clone platform that is patterned after coronavirus

systems and describe additional details including transcript infectivity and recombinant virus phenotypes *in vitro* (20, 85, 86, 149, 150). Generating a panel of contiguous cDNAs that span the entire genome, the divided DENV1 reverse genetic system overcomes toxic elements within itself and allows propagation in bacteria. Importantly, directional assembly and *in vitro* transcription allows electroporation of full length infectious RNA that directly corresponds to the wild-type virus sequence. As a result, the infectious clone derived virus maintains similar replication in multiple cell types (Figure 5.1E-G) as well as complete fidelity in regard to antibody binding and virus neutralization (Figure 5.2 and 5.3). Building on previous epitope swap mutants (20, 21), we employed this reverse genetic system to generate a DENV1 viral mutant that displayed a known monoclonal antibody epitope from DENV4, gaining 5H2 monoclonal antibody binding and neutralization to the donor sequence strain. However, the mutant virus also disrupted binding and neutralization of two DENV1 specific monoclonal antibodies despite retaining the majority of their targeted antigenic residues (Figure 5.5 and 5.7). While other DENV1-specific monoclonal antibodies maintain effective binding and neutralization (Figure 5.7C), the data highlight the opportunity cost of domain swaps even in the context of partial epitope disruption. Overall, the results also illustrate the utility of this newly developed infectious clone systems as a platform to study DENV1 infection, pathogenesis and immunity.

For the DENV vaccine field, this reverse genetic system amplifies opportunities in virus design that are already being explored. Previous work by our lab has utilized the DENV2 and DENV4 infectious clones to define and transfer a critical antibody binding epitope in DENV2 identified by structural analysis (21, 38). In this case we described a recombinant DENV4 virus that displayed a heterologous DENV2 epitope, while preserving the major neutralizing epitopes on DENV4 (21). In addition, previous work using this DENV1 infectious clone was able to transfer part of the DENV3 MAb 5J7 epitope, resulting in a partial gain of binding and neutralization by 5J7, but no loss of binding to DENV1 MAb 1F4 (20). Importantly, both the

DENV2 2D22 and DENV3 5J7 MAb epitopes are distinct from the 1F4 and 14C10 epitopes transplanted here. Coupled with data from the current manuscript, the approach to disrupt epitopes within the context of live virus highlights an independent and powerful approach to quickly validate structural predictions of key residues. Importantly, full characterization of these viral epitopes and their portability between DENV serotypes opens new approaches to vaccine development. Importantly, this work defines a rationally-designed chimeric virus that uses MAb-envelope structural interactions to identify residues associated with the DENV4 MAb 5H2 epitope that were sufficient for the gain of binding and neutralization. These changes lead to the disruption of multiple overlapping DENV1 epitopes, highlighting a potential problem that must be addressed in DENV vaccine design. Interestingly, despite maintaining nearly two thirds of the DENV1 1F4 epitope in the chimeric virus, binding and neutralization were almost completely lost, indicating that the critical residue determinants for antibody interactions were among those that were changed. Another DENV1 MAb, 14C10, which maintained a much larger percentage of its epitope in the chimeric virus, lost a smaller amount of binding, yet still lost nearly complete neutralization by 14C10. This suggests that while the remaining epitope can still be partially bound by 14C10, the interaction is insufficient to fully neutralize the virus. Structure-guided design could identify additional residues to further expand the transplanted DENV4 epitope to fully ablate DENV1 MAb and polyclonal binding and neutralization. Together, these data illustrate the utility of mapping epitopes using a combination of structure and reverse genetics. With a similar approach employed for human norovirus (151-155), these resources can be applied to generate diagnostic reagents, and to create a map of epitopes ideal for designing chimeric bivalent vaccines that produce broad-spectrum neutralization, a critical issue in DENV pathogenesis.

Moving beyond vaccine immunity, the infectious clone system provides opportunities to contribute to other areas of DENV research. Exploring virus-host interactions has been

complicated by attenuation of traditional clone derived virus (134). Our system maintains equivalent replication to the wild-type strain, allowing examination of the impact of specific point mutations or viral protein deletion without the complication of interpreting baseline clone attenuation. In addition to these areas, the infectious clone system may be a useful resource in identifying virulence determinants, host specificity, viral protein function, RNA structural elements as well as testing of antiviral therapeutics.

Overall, the reverse genetic system characterized in this manuscript represents a major resource for the study of DENV1 infection and pathogenesis. Building on previous reports (20), this clone system provides several important advantages to study DENV1 including robust propagation in bacteria, fidelity to wild-type sequence, and absence of replication attenuation. Importantly, both antibody binding and virus neutralization indicate uniform antigenicity with the wild-type strain permitting epitope mapping. Coupled with structural studies, the reverse genetic system can be manipulated to produce chimeric vaccines harboring conserved epitopes from multiple serotypes. While it is unlikely that neutralizing epitopes from all four serotypes could be combined into a single viable chimeric DENV virus, it is possible two bivalent viruses could be generated that contain neutralizing epitopes from all four serotypes (e.g. rDENV1/3 and rDENV4/2). This advance offers a promising strategy to produce broad based protection against the DENV family.

## **5.6 Materials and Methods**

### *Virus Construction*

Recombinant DENV1 West Pac '74 infectious clone (rDENV1ic) was constructed using a four-cDNA cloning strategy as previously described (20, 21, 87). Briefly, the DENV1 genome was divided into four fragments, and subcloned into separate cDNA plasmids with unique type IIS restriction endonuclease cleavage sites at the 5' and 3' ends of each fragment. A PflMI site



was removed using standard molecular techniques. A T7 promoter was introduced into the 5' end of the A fragment, and plasmid DNA was grown in *Escherichia coli* cells. Purified plasmid DNA was enzyme digested, purified, and ligated together with T4 DNA ligase. Infectious genome-length capped viral RNA transcripts were generated with T7 polymerase. RNA was electroporated into C6/36 cells and incubated 4-6 days. Cell culture supernatant containing virus was harvested, centrifuged at maximum speed to remove cellular debris, and passaged onto C6/36 cells to generate a passage one virus stock.

#### *rDENV1ic-EDI Mutant Construction*

For mutant virus generation, 12 predicted predicted contact residues of 5H2 (80, 147) were identified that differ between DENV1 and DENV4 (Table 1). rDENV1ic plasmid A fragment was re-designed to encode these 12 amino acid changes in the envelope protein, to create rDENV1ic-EDI. The new A plasmid was digested and ligated with WT rDENV1ic plasmid B, C and D, as described above, to generate the rDENV1ic-EDI mutant virus.

#### *Cells*

Cells were cultured as previously described by our group (20, 21). C6/36 cells were grown in Gibco minimal essential medium (MEM) at 32°C. DC-SIGN expressing U937 cells (U937+DC-SIGN) were maintained in RPMI-1640 at 37°C. Media were supplemented with fetal bovine serum (FBS) (5% for C6/36 and U937+DC-SIGN cells) which was lowered to 2% after infection. C6/36 and U937+DC-SIGN media were supplemented with nonessential amino acids, and U937+DC-SIGN medium were also supplemented with L-glutamine and 2-mercaptoethanol. All media were additionally supplemented with 100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml Amphotericin B. All cells were incubated in 5% CO<sub>2</sub>.

### *Virus Titration and Immunostaining*

24-well cell culture plates were seeded with C6/36 cells to be confluent at time of infection. Cell growth media was removed, virus stocks were serially diluted 10-fold then added to cells for one hour at 32°C (C6/36) with gentle rocking. After incubation, cells were overlaid with 1% methylcellulose in OptiMEM I (Gibco) supplemented with 2% FBS, nonessential amino acids and 100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml Amphotericin B, and incubated at 32°C. After four-six days incubation, overlay was removed, cells were washed with phosphate-buffered saline (PBS) and fixed in 80% methanol. Cells were blocked in 5% non-fat dried milk in PBS (blocking buffer) then incubated for 1 hour at 37°C with anti-prM MAb 2H2 and anti-E MAbs 4G2 and DV1-E103 diluted in blocking buffer. Cells were washed two times with PBS, then incubated for 1 hour at 37°C with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Sigma) diluted in blocking buffer. Plates were washed two times with PBS, and foci were developed using TrueBlue HRP substrate (KPL).

### *Infectious Center Assay*

C6/36 cells electroporated with rDENV1ic RNA were diluted in OptiMEM I (Gibco), added to a confluent monolayer of C6/36 cells, overlaid with 1% methylcellulose and incubated for four days. After incubation, cells were fixed and stained as described above. Percent of electroporated cells capable of making viable infectious virus was calculated as follows = (number foci/number of ex electroporated cells plated) x 100.

### *Growth Curves*

To determine the amount of virus cells can produce, C6/36 cells were inoculated at a multiplicity of infection (MOI) of 0.01. Every 24 hours, all cell culture supernatant was harvested (volume was replaced with fresh medium) and frozen at -80°C. Virus samples were titered as described above. To determine the kinetics at which cells become infected, C6/36 cells were

inoculated at an MOI of 0.01. Every 12 hours, cell culture medium was removed, cells were washed with PBS, fixed, permeabilized and probed with anti-prM MAb 2H2 conjugated to AlexFluor488. Infected cells were quantified using a Guava flow cytometer (Millipore). U937+DC-SIGN cells were infected at an initial infection of 2%, and every 12 hours a sample of cells were harvested, fixed and stained as described for C6/36 cells.

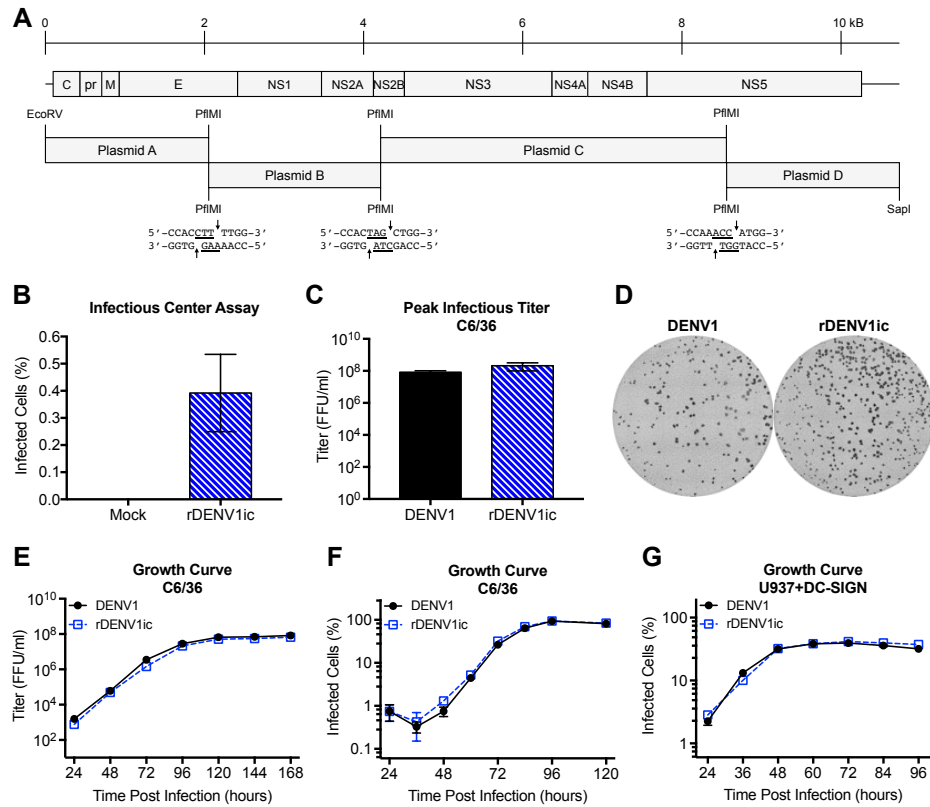
#### *Binding Enzyme-Linked Immunosorbent Assay (ELISA)*

Plates were coated with either 100ng/well each mouse MAbs 4G2 and 2H2, or 200ng/well human MAb 1C19, overnight at 4°C. Plates were washed with Tris-buffered saline with 0.05% Tween (TBST) and blocked in 3% non-fat dried milk in TBST (blocking buffer), and equal quantities of virus (as previously titrated by ELISA) were added and incubated for 1 hour at 37°C. Plates were washed and primary human MAbs were diluted 4-fold in blocking buffer and added to plate for 1 hour. Plates were washed and alkaline phosphatase (AP)-conjugated secondary antibodies were added for 1 hour at 37°C. Plates were washed, developed using *p*-nitrophenyl phosphate substrate and color changes were quantified by spectrophotometry as previously described (20, 21).

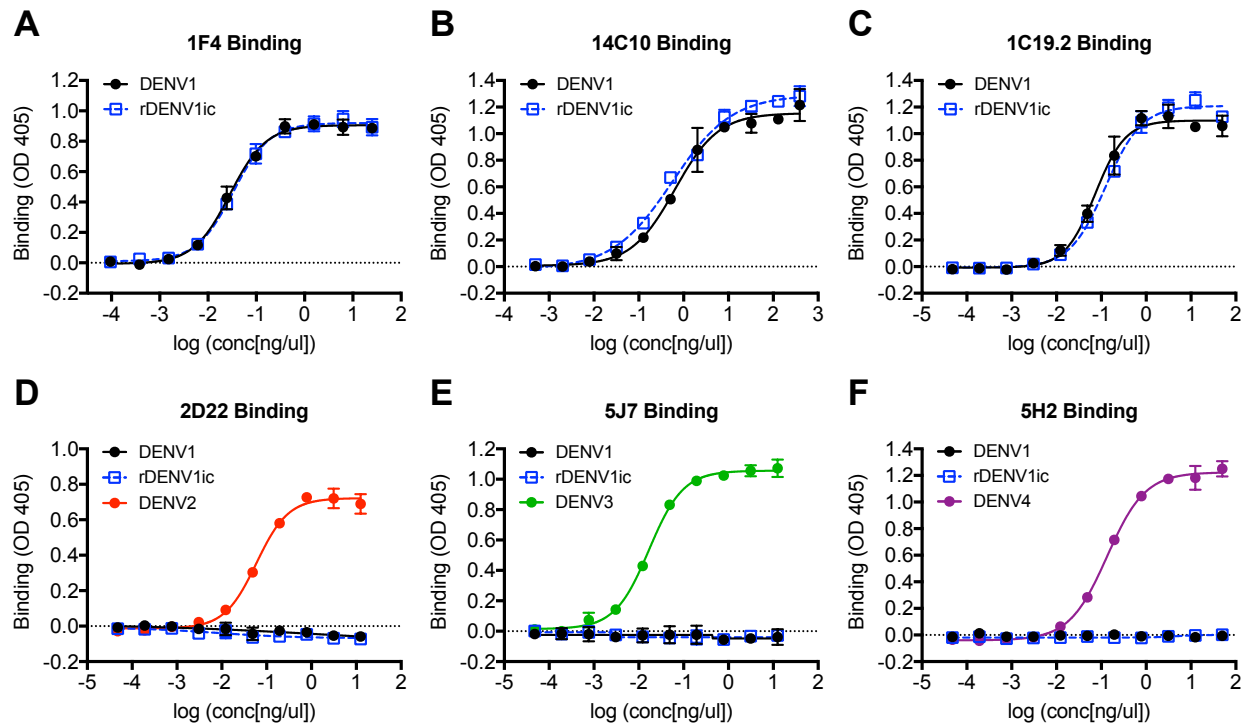
#### *Neutralization Assays*

For the focus reduction neutralization test (FRNT), 24-well cell culture plates were seeded with C6/36 cells to be confluent at time of infection. MAbs were diluted 4-fold and mixed with ~45 focus-forming units (FFU) of virus, and incubated for 1 hour at 32°C. After incubation, virus:MAb mixture was added to C6/36 cells for 1 hour at 32°C with gentle rocking. Overlay was added and cells were incubated 4-6 days. Cells were fixed and stained as described above. For the flow-based neutralization assay, MAbs were diluted 4-fold and mixed with virus (previously titrated to equal ~15% infection with no MAb present), and incubated for 1 hour at 37°C. After incubation, virus:MAb mixture was added to  $5 \times 10^4$  U937+DC-SIGN cells for 2 hours at 37°C.

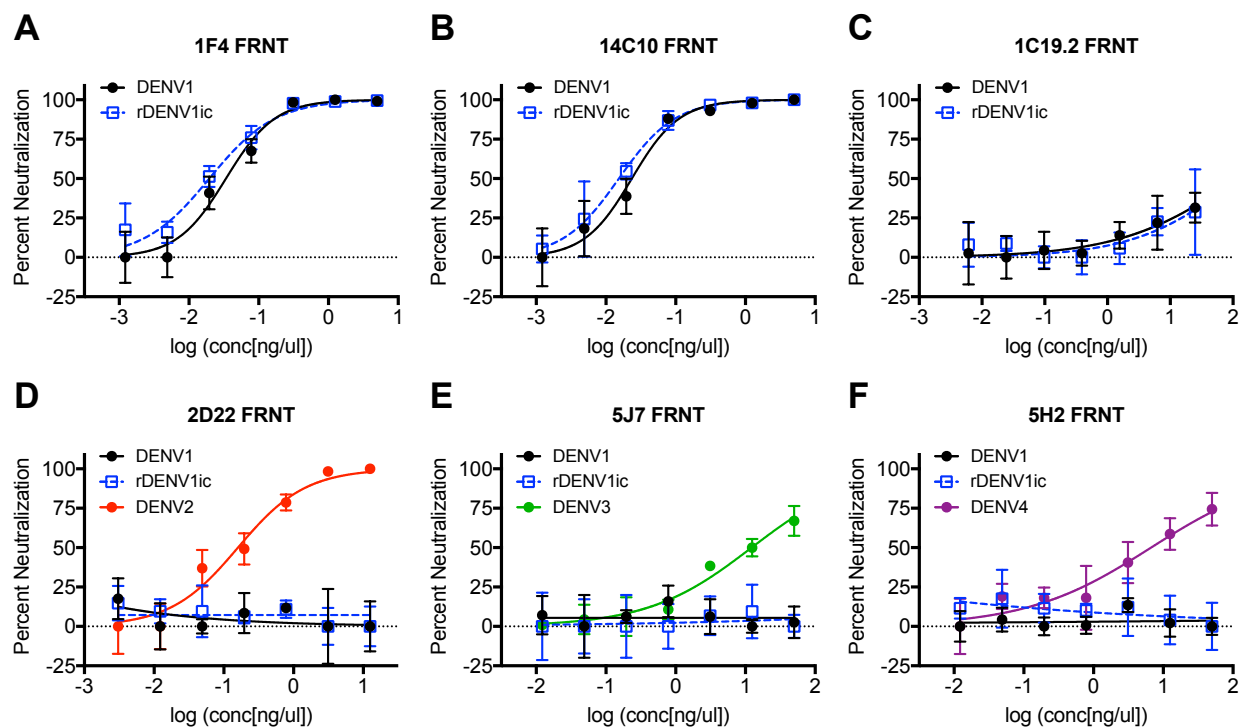
Cells were then pelleted and washed two times with fresh medium, then incubated for 24 hours. After incubation, cells were fixed, permeabilized and stained as described above for U937+DC-SIGN growth curves.



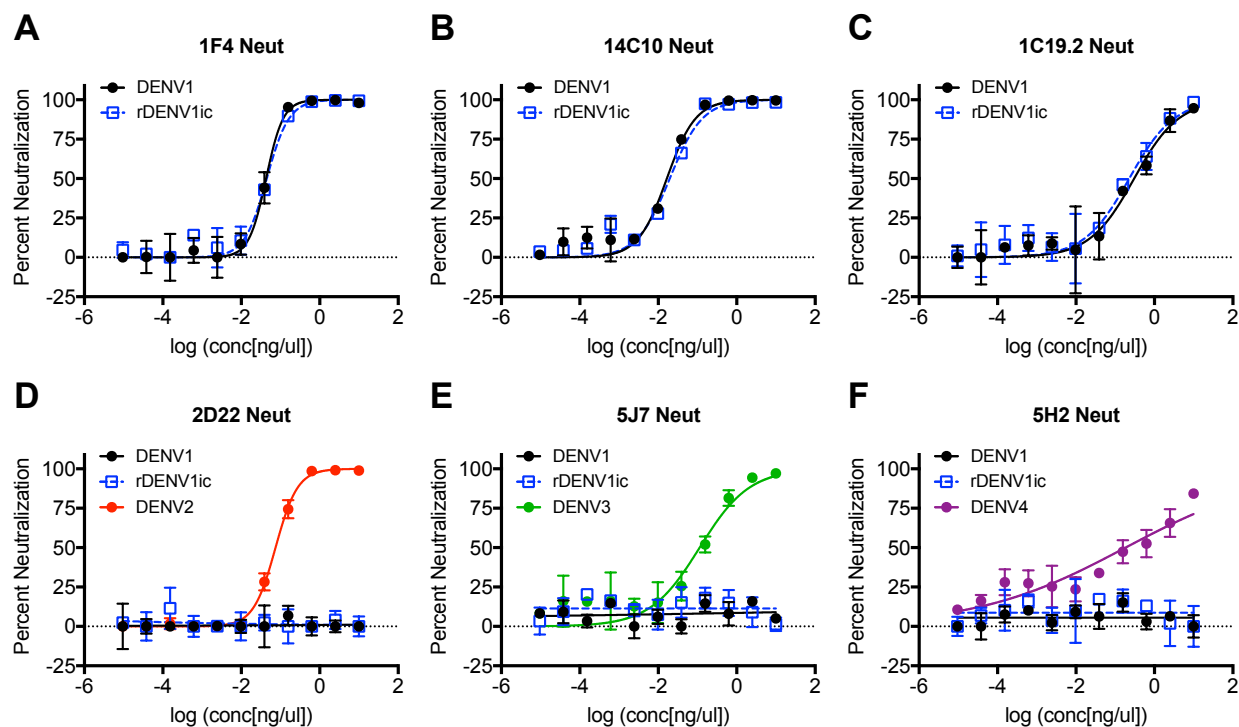
**Figure 5.1. DENV1 infectious clone design, characterization and growth.** (A) Structure of DENV1 viral genome and division into the four subgenomic fragments designated plasmids A (nucleotides 1-2052), B (2053-4215), C (4216-8563) and D (8564-10736). (B) Full-length viral RNA was electroporated into C6/36 cells, cells were diluted and added to confluent C6/36 cells. After incubation, infected cells were stained to determine initial percentage of electroporated cells capable of producing infectious virus. (C) Peak infectious titers of virus stocks were determined on C6/36 cells. (D) Immunostaining of DENV1 and rDENV1ic foci in C6/36 cells. (E) C6/36 cells were inoculated at an MOI of 0.01 and every 24 hours, supernatant containing virus was harvested and subsequently titrated on C6/36 cells. (F) C6/36 cells were inoculated at an MOI of 0.01, or (G) 1% of U937+DC-SIGN cells were infected, and every 12 hours, cells were harvested, stained for intracellular viral antigen, and total percentage of infected cells was calculated.



**Figure 5.2. rDENV1ic is only bound by DENV1-serotype specific antibodies.** DENV1 and rDENV1ic were analyzed for their ability to bind (A-C) DENV1-serotype specific antibodies 1F4, 14C10, and 1C19.2 or (D-F) DENV2, DENV3 and DENV4-serotype specific antibodies, 2D22, 5J7 and 5H2, respectively, in an ELISA virus capture assay.



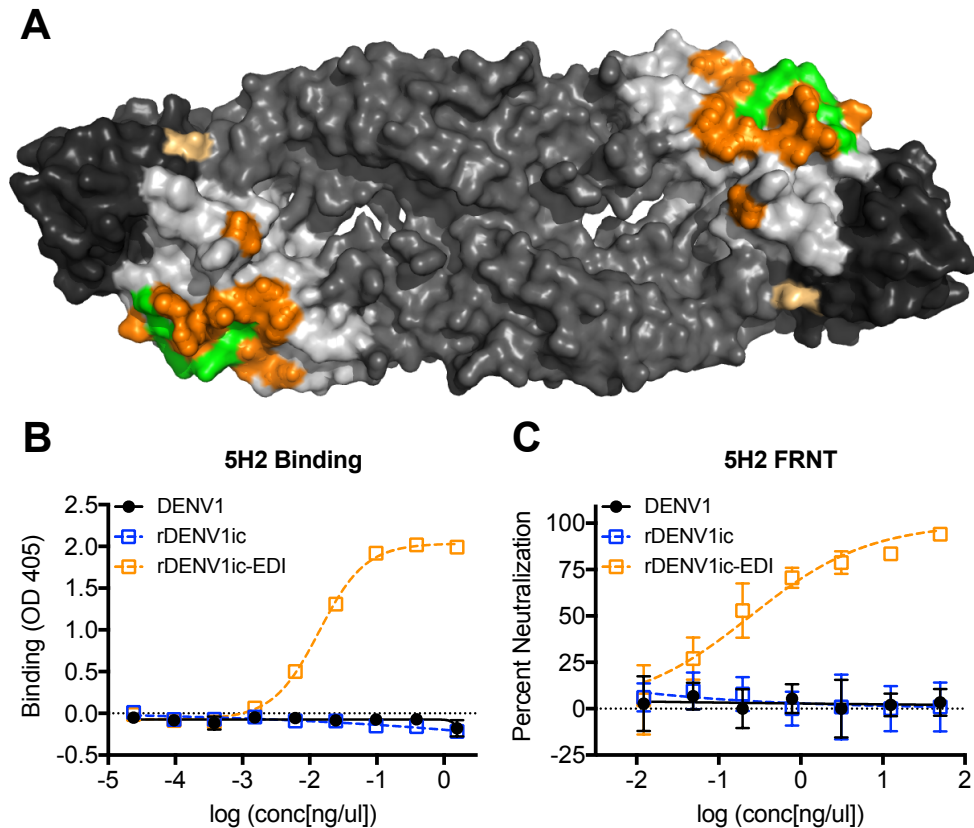
**Figure 5.3. rDENV1ic maintains fidelity to DENV1-serotype specific antibodies in a focus reduction neutralization test.** Neutralization of DENV1 and rDENV1ic by monoclonal antibodies was measured in a C6/36 focus reduction neutralization test (FRNT) using (A-C) DENV1-serotype specific MAbs 1F4, 14C10 and 1C19.2 and (D-F) DENV2, DENV3 and DENV4-serotype specific antibodies, 2D22, 5J7 and 5H2, respectively.



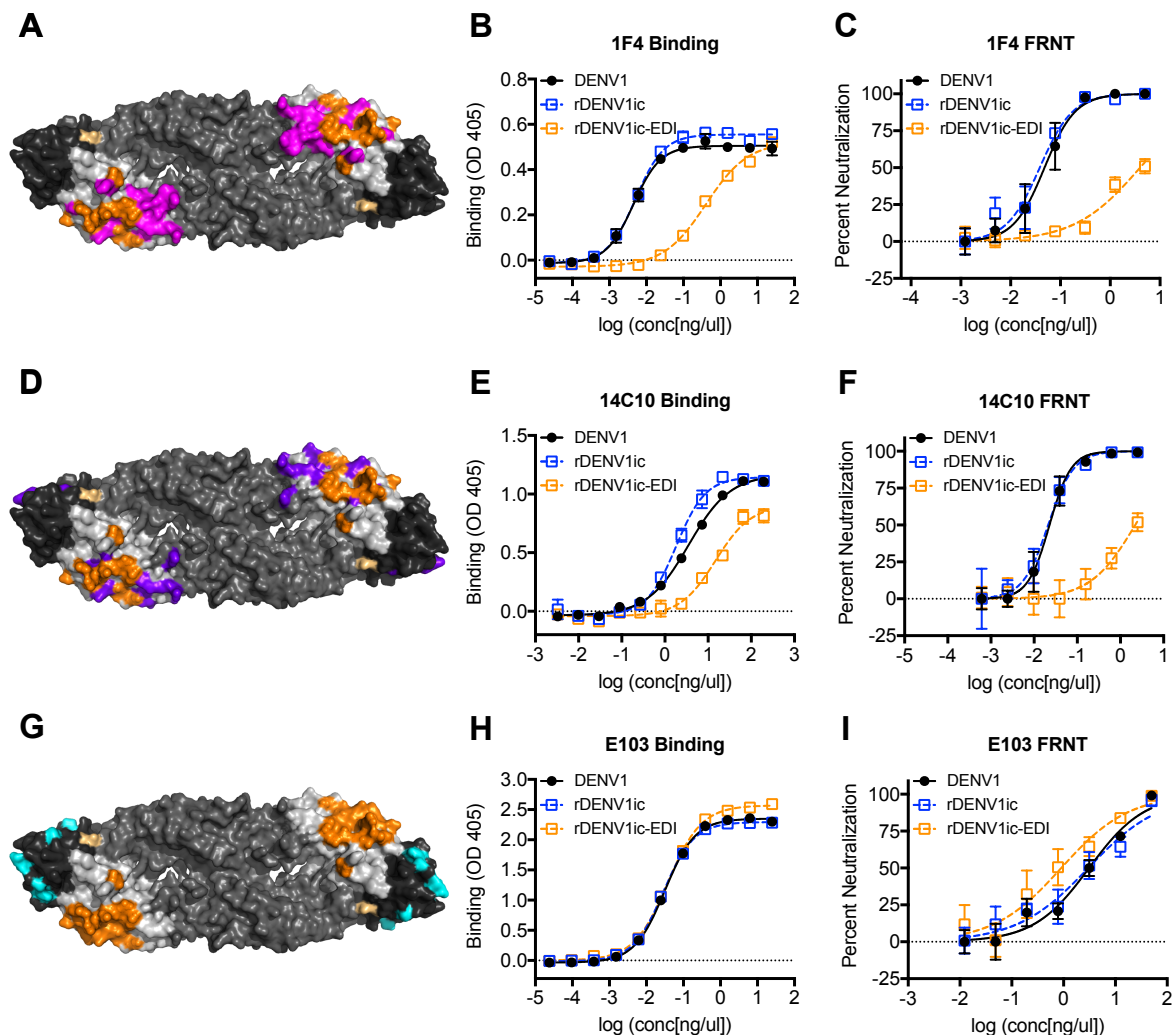
**Figure 5.4. rDENV1ic is only neutralized by DENV1-serotype specific antibodies in a flow-cytometry based neutralization test.** Neutralization of DENV1 and rDENV1ic by monoclonal antibodies was measured in U937+DC-SIGN flow-cytometry neutralization assay using (A-C) DENV1-serotype specific MAbs 1F4, 14C10 and 1C19.2 and (D-F) DENV2, DENV3 and DENV4-serotype specific antibodies, 2D22, 5J7 and 5H2, respectively.



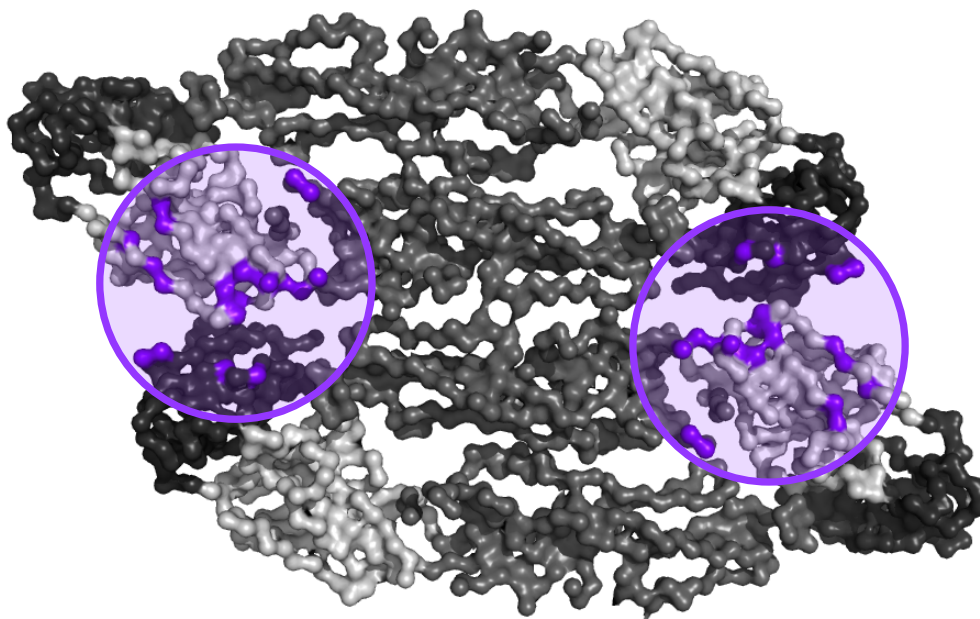




**Figure 5.6. rDENV1ic-EDI gains binding and neutralization to DENV4-serotype specific MAb 5H2.** (A) Structure of DENV envelope protein dimer showing transplanted residues from DENV4 (orange) and MAb 5H2 epitope contact residues (green). Residues that are part of both the 5H2 MAb epitope and rDENV1ic-EDI virus are shown as orange. rDENV1ic-EDI virus that was recovered contained an additional mutation (light orange) unrelated to the transplanted 5H2 epitope. (B) Viruses were analyzed in an ELISA virus capture assay for their ability to bind DENV4-specific MAb 5H2. (C) Neutralization of viruses in C6/36 focus reduction neutralization test (FRNT) with DENV4-specific MAb 5H2.



**Figure 5.7. rDENV1ic-EDI loses binding and neutralization to EDI DENV1-serotype specific MAbs.** Structure of DENV envelope protein dimer showing transplanted residues from DENV4 (orange) and MAb epitope contact residues for (A) 1F4 (magenta), (D) 14C10 (purple) and (G) E103 (cyan). Residues that are part of both the MAb epitope and rDENV1ic-EDI virus are shown as orange. rDENV1ic-EDI virus that was recovered contained an additional mutation (light orange) unrelated to the transplanted 5H2 epitope. Viruses were analyzed in an ELISA virus capture assay for their ability to bind MAbs (B) 1F4, (E) 14C10 and (H) E103. Neutralization of viruses in C6/36 focus reduction neutralization test (FRNT) with MAbs (C) 1F4, (F) 14C10 and (I) E103.



**Figure 5.8. 14C10 epitope spans into EDIII from neighboring dimer.** The 14C10 epitope sits primarily over EDI of a single envelope dimer, but it interacts with additional residues of EDIII in the adjacent envelope dimer.

Serotype-Specificity	MAb	Species	Epitope
DENV1	1F4	Human	EDI, EDI/II hinge
	14C10	Human	EDI, EDI/II hinge
	1C19.2	Human	EDIII
	E103	Mouse	EDIII
DENV2	2D22	Human	EDIII/EDII
DENV3	5J7	Human	EDI/II hinge
DENV4	5H2	Human	EDI

**Table 5.1. Monoclonal antibody serotype-specificity and epitopes.** Serotype-specificity, species and epitopes of MAbs used in experiments

## **CHAPTER 6 – Discussion**

### **6.1 Overview of Human Antibody Response to Dengue Infection**

The last ten years has seen a shift in the field's understanding of human DENV antibodies (66, 67). Decades of prior research using anti-DENV mouse monoclonal antibodies was critical to our basic understanding of DENV-MAb interactions and virus biology, but importantly, did not completely inform our understanding of the human antibody response. While mice generate neutralizing antibodies to an immunodominant, simple EDIII epitope, humans target different regions encompassing more complex epitope structures that span monomers or are dependent on particle morphology (64). This shift has not only placed a new emphasis on the importance of studying human monoclonal and polyclonal antibodies to better understand the human adaptive immune response to DENV infection, but also provides new leads for the development of therapeutics. Importantly, with our growing knowledge on this subject, our metrics of antibody-based protection have changed greatly. My PhD research substantially contributes to our current understanding of the human antibody response to DENV infection and vaccination. Specifically, I defined major antigenic sites on DENV2 targeted by strongly neutralizing antibodies produced by memory B cells and long-lived plasma cells in people exposed to natural infections or vaccines. While polyclonal serum antibody responses to DENVs are known to be a complex mixture of different specificities, my studies indicate that the functionally neutralizing response was relatively simple and directed to one or two immunodominant sites. My work has been used to evaluate vaccine responses to DENV infection, and better inform the design of next-generation vaccines (22, 156-158).

For many viruses, the mere presence of virus-specific antibodies is a strong correlate of protection, and thus, many vaccines have been developed and evaluated by their ability to elicit high titers of antibodies. Following a natural DENV infection, individuals generate large populations of specific IgG antibodies (64). These antibodies are comprised of a population of serotype-specific neutralizing antibodies that are specific to the infecting serotype, but importantly, there is also a population of cross-reactive non-neutralizing antibodies (12). Therefore, despite being immune to only the infecting serotype, and susceptible to reinfection with any of the three remaining serotypes, individuals would test positive for the presence of antibodies reactive to all four serotypes (12). This highlights the difference between DENV and many other human virus pathogens, reveals the complexity of the adaptive immune response to flaviviruses, and complicates the use of the presence of anti-DENV antibodies as a correlate for protection.

## **6.2 My Contributions to the DENV Antibody Field**

To better understand the properties of human antibodies that protect people from DENV infections, I studied both the quantity and quality of neutralizing antibodies in DENV-naïve individuals that were naturally infected with DENV (primary infections) or vaccinated with monovalent or tetravalent DENV vaccines. In my research I used antibody depletion methods and developed novel recombinant DENVs to characterize the molecular specificity and functional properties of different antibody populations in human immune sera. While previous studies with human DENV-immune sera mainly focused on measuring absolute levels of neutralizing antibodies, my results reveal new layers of information about the molecular properties the human antibody response to DENV infection. Recent studies indicate that the mere presence of DENV neutralizing antibodies is not sufficient for protection (68, 69). I propose that metrics that consider both the molecular specificity and level of neutralizing antibodies are

likely to be better correlates of protection than current metrics based on the presence or level of neutralizing antibody.

Basic research on the properties of human DENV monoclonal antibodies has identified critical epitopes for each serotype targeted by both monoclonal and polyclonal antibodies (20, 23, 27, 35, 37, 38, 87, 103, 126, 159). My research has focused on describing the properties of human DENV1 and DENV2 monoclonal and polyclonal antibodies, and the epitopes they target on the DENV envelope protein (103, 126, 159). My work with DENV1 has shown that multiple strongly neutralizing human antibodies target a complex EDI epitope (103). Importantly, while the epitopes of these antibodies are overlapping, the specific residues on the envelope that the antibody interacts with vary. This reveals that there is not a single epitope, but instead of broader region that likely contains and encompasses the epitopes of many monoclonal and polyclonal antibodies. Additionally, I have shown that there is plasticity within the envelope protein, and that these neutralizing epitopes can be transplanted between serotypes, while maintaining their functionality (103).

My research studying immunity to DENV2 infection has revealed that there are two dominant neutralizing quaternary epitopes (126, 159). I found that the epitopes defined by monoclonal antibodies from two individuals, are representative of polyclonal antibody neutralizing epitopes across a range of different individuals exposed to natural infections or live attenuated vaccines, however the specific levels of these antibodies vary among individuals. Like DENV1, these epitopes are transferrable between serotypes, resulting in chimeric viruses that contain these functional, neutralizable epitopes. My work defining individual antibodies epitopes, generating recombinant viruses that capture those epitopes, and using these viruses as tools to map polyclonal antibody epitopes has advanced our ability to dissect the properties of the complex human antibody response to DENV infection and vaccination.



These types of experiments add yet another parameter to evaluate antibody-based correlates of protection, by determining the epitopes targeted by serotype-specific antibodies (67). Currently, the best correlate of protection against DENV infection is the presence of high levels of neutralizing antibodies (66, 67). However within this broad metric, we know very little about the properties of these antibodies that are necessary to provide protection, such as their absolute level, the amount that are serotype-specific, or the epitopes that they target. Whether anti-DENV antibodies protect by providing sterilizing immunity upon subsequent infection, or through another mechanism is unknown. Using techniques, tools, and results generated from my thesis research, the field can begin to answer these questions.

### **6.3 Implications of DENV Antibody Immunity, Vaccines and Enhanced Disease**

In addition to strongly neutralizing antibodies, people exposed to primary infections generate serotype cross-reactive antibodies, which are poorly neutralizing and not correlated with durable protective immunity (12). These antibodies have been implicated in enhancing disease during secondary infections via a theory known as antibody dependent enhancement (ADE) (13). The ADE theory postulates that cross-reactive and poorly neutralizing antibodies enhance infection by forming Ab:virus immune complexes that are infectious to some Fc-receptor bearing human cells. The concentration of cross-reactive antibody required for ADE to occur in people is unknown, but it is thought that it is most likely to occur when these antibody levels are relatively low (13). Indeed, recent analyses have revealed that during naturally acquired DENV infections, high levels of antibodies protect against severe disease, but ADE only occurs at a low, narrow range of pre-existing anti-DENV antibody titers (89). As human polyclonal DENV antibodies are important in not only protection, but also enhanced disease, it is critical that we fully define their properties to better understand natural infection, but also to evaluate DENV vaccines.

An effective DENV vaccine should result in an independent, robust antibody response to all four serotypes simultaneously (3, 71, 73, 97, 160). If the immune response is unbalanced, it can result in populations of antibodies that enhance a natural infection via the mechanism of antibody dependent enhancement (ADE) (13). Indeed, this unfortunately appears to be the case with Sanofi Pasteur's live attenuated tetravalent DENV vaccine, Dengvaxia (68, 121, 122). Since non/poorly-neutralizing cross-reactive antibodies are implicated in causing enhanced disease via ADE, it is critical that an effective vaccine either does not elicit this population of antibodies, or is able to generate large populations of strongly neutralizing antibodies to provide enough sterilizing immunity so that enhancement cannot occur. Using depletion techniques and other assays, and recombinant viruses that I have developed and characterized, we can distinguish between these different types of enhancing and neutralizing antibodies, and evaluate the likelihood of enhanced disease following vaccination.

#### **6.4 Overview of Progress and Pitfalls of DENV Vaccines**

As our understanding of antibody-based immunity to DENV infection has evolved, the metrics used to evaluate vaccines has changed as well. Using the MAbs, recombinant viruses and reagents that I have generated, we have evaluated the antibody response to multiple DENV vaccine platforms to compare vaccine-elicited antibody-based responses with those from natural DENV infections. Early pre-clinical and clinical results from Dengvaxia trials, revealed that the vaccine elicited high levels of neutralizing antibodies that neutralized all DENV serotypes, suggesting individuals would be protected (68). Breakthrough infections in individuals with high levels of neutralizing antibodies revealed that the mere presence of these antibodies is not an accurate correlate of protection (68, 69). More recent evaluation of the quality of response following vaccination has revealed that although there is cross-neutralization of all serotypes, this is not primarily due to serotype-specific antibodies, which are known to be critical for protection (22). Rather, the vaccine elicits cross-reactive neutralizing responses suggesting

that qualitative differences in adaptive immune targeting can affect vaccine performance. Using results I've discussed, through studying DENV antibodies following natural infection, we can continue to improve design of next-generation DENV vaccines and more accurately evaluate them (66, 67).

## **6.5 Comparison to Other Viruses**

My PhD work has defined multiple human DENV monoclonal antibodies and the quaternary epitopes that they target (103, 126, 159). The assays and techniques used to identify the properties of anti-DENV antibodies are applicable to many other viruses as well (161). With the emergence of Zika virus (ZIKV) in 2015, while some groups focused on generating mouse antibodies, other groups were able to rapidly isolate and generate strongly neutralizing human antibodies (29-31). This was aided using assay developed and optimized in the DENV field, especially the use of techniques that allow for detection of antibodies recognizing quaternary epitopes (29-31). Indeed, like DENV and West Nile Virus (WNV) (27, 32), the potentially neutralizing antibodies responsible for human polyclonal neutralization of ZIKV, target many of the same quaternary epitopes as previously identified for these other flaviviruses. Decades of research on DENV antibodies enabled the field to quickly identify these critical antibodies, and harness them for diagnostic, vaccine and therapeutic applications (161, 162).

Quaternary epitopes targeted by human neutralizing antibodies is not limited to the flavivirus field. More recently it has been found that many human neutralizing antibodies against both Marburg and Ebola viruses target quaternary epitopes on the virus glycoproteins (163, 164). These epitopes span across monomers within the trimeric glycoprotein, and have been suggested to neutralize virus by either blocking receptor interactions or locking proteins and preventing conformational changes required for infection (163, 164). These exact mechanisms have been shown with DENV quaternary epitope antibodies (160, 161), suggesting these are

potentially conserved mechanisms across diverse virus families. Indeed, human antibodies targeting quaternary epitopes have been identified for HIV, influenza, norovirus and likely will be identified for many other viruses as well (165-168).

## **6.6 Future Directions for the Flavivirus Field**

Results presented in this dissertation have contributed to expanding our understanding of the human antibody response to both natural DENV infection and vaccination. Additionally, simultaneous work defining other aspects of DENV immunity, including innate immune responses (169), T-cell components (96), and viral determinants of severe disease (170), has further refined our understanding of the complete immune response to human DENV infection.

Despite these advances made in DENV immunology research over the last decade, the failure of Sanofi Pasteur's Dengvaxia vaccine, has presented a challenge to our field. Unlike many viral pathogens, development of a successful DENV vaccine has been complicated by the risk of incomplete protection leading to more severe disease due to ADE (3, 72, 73). Given these concerns, DENV vaccine progress has been especially slow, with extensive pre-clinical and clinical testing (73). Despite this decades-long process of vaccine development, many of the issues seen with Dengvaxia were unexpected considering what was known at the time, and have highlighted critical gaps in understanding DENV immunity. Specifically, we still do not know the locations of all neutralizing epitopes for each serotype, or for cross-neutralizing antibodies that are generated after secondary infections. Additionally, it is unclear what epitopes enhancing antibodies target, and if these vary between the four DENV serotypes. As we further define these antibodies and their epitopes, we will be closer to generating antibody-based correlates of protection, which will be critical in evaluating DENV vaccines. In addition to anti-E antibodies, the role of antibodies directed at the NS1 protein, along with T-cell immunity is not fully known, but is likely also part of a protective immune response to DENV infection.

Knowing what we know now from my studies and the work of others, the failure of Dengvaxia in naïve individuals was predictable. While vaccination elicited high levels of neutralizing antibodies to all four serotypes, it is now clear that the quality of the antibodies might not have been protective, but instead, enhancing (4, 22, 68, 121). The new insights and tools generated from my work can be used in preclinical studies to evaluate vaccine efficacy in naïve populations, beyond the mere presence of neutralizing Abs. There were other surprises from the phase III clinical trials that we did not expect however, such as the drastic differences in vaccine efficacy against different genotypes within a single serotype, namely for DENV4 (130). This highlights the fact that, despite the rapid advances the field has collectively made, there are aspects of DENV immunology that we haven't yet begun to understand. My recent work has looked at the role of genotypic variation within DENV4. We find that in evaluating vaccine responses, it is critical to study multiple genotypes, especially those currently circulating in human populations and causing disease, to fully capture the vaccine-elicited antibody response.

Sustained fundamental research of both the virus and the host will continue to elucidate the complex relationship between DENV and the hosts it infects. In addition, research understanding other aspects of DENV, including its vector, epidemiology, clinical manifestations, and reservoir hosts, will further fill in our existing knowledge gaps.

## APPENDIX

In addition to the work discussed in this dissertation, I have contributed to other published and unpublished projects, detailed below. These have been in collaboration with talented scientists both at UNC and other institutions.

In collaboration with Kenneth Dinno III, we performed and published a study investigating the role of the Zika virus envelope protein's contribution to virus stability.

**Gallichotte EN**, Dinno KH III, Lim XN, Ng TS, Lim EXY, Menachery VD, Lok SM, Baric RS. "CD-loop extension in Zika virus envelope protein key for stability and pathogenesis". J Infect Dis. 2017 Dec 5;216(10):1196-1204

As briefly discussed in chapter 4, I have completed a project investigating the role of genotypic variation within DENV serotype 4, on many aspects of virus biology, and virus antibody interactions.

**Gallichotte EN**, Baric TJ, Nivarthi U, Graham R, Widman DG, Yount BL, Durbin A, Whitehead S, de Silva AM, Baric RS. "Genetic variation between dengue virus type 4 strains impacts human antibody binding and neutralization". *Under review*

I assisted with the development and characterization of Zika virus infectious clones, resulting in the two following publications.

Widman DG, Young E, Yount BL, Plante KS, **Gallichotte EN**, Carbaugh DL, Peck KM, Plante J, Swanstrom J, Heise MT, Lazear HM, Baric RS. "A Reverse Genetics Platform That Spans the Zika Virus Family Tree". MBio. 2017 Mar 7;8(2)

Swanstrom JA, Plante JA, Plante KS, Young EF, McGowan E, **Gallichotte EN**, Widman DG, Heise MT, de Silva AM, Baric RS. "Dengue Virus Envelope Dimer Epitope Monoclonal Antibodies Isolated from Dengue Patients are Protective Against Zika Virus". MBio. 2016 Jul 19;7(4)

I contributed to experimental design, data generation, data analysis, and writing and editing for the four following papers.

Nivarthi UK, Kose N, Sapparapu G, Widman D, **Gallichotte E**, Pfaff JM, Doranz BJ, Weiskopf D, Sette A, Durbin AP, Whitehead SS, Baric R, Crowe JE Jr, de Silva AM. "Mapping the Human Memory B Cell and Serum Neutralizing Antibody Responses to DENV4 Infection and Vaccination". *J Virol*. 2017 Feb 14;91(5)

Liu P, Ridilla M, Patel P, Betts L, **Gallichotte E**, Shahidi L, Thompson NL, Jacobson K. "Beyond attachment: roles of DC-SIGN in dengue virus infection". *Traffic*. 2017 Apr;18(4):218-231

Metz SW, **Gallichotte EN**, Brackbill A, Premkumar L, Miley MJ, Baric R, de Silva AM. "In Vitro Assembly and Stabilization of Dengue and Zika Virus Envelope Protein Homo-Dimers". *Sci Rep*. 2017 Jul 3;7(1):4524

Swanstrom J, Henein S, Plante J, Yount B, Widman D, **Gallichotte EN**, Dean H, Osorio JE, Parditos CD, de Silva AM, Baric RS. "Analyzing the human serum antibody responses to a live attenuated tetravalent dengue vaccine (TDV) candidate". *J Infect Dis*. *In press*

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